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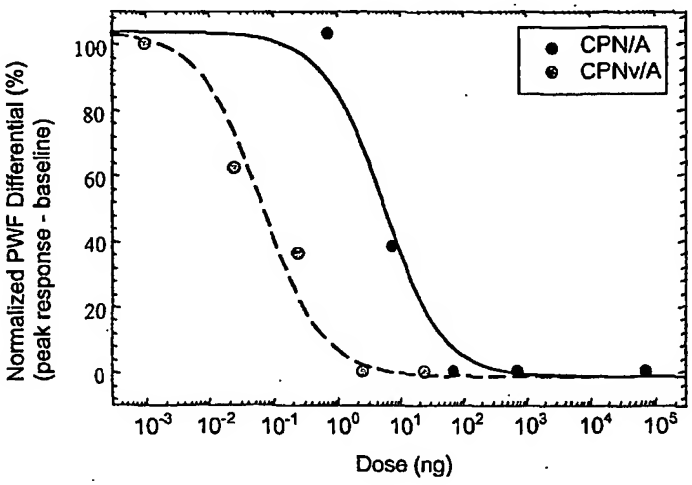

(57) Abstract: Use of a therapeutic molecule, for the treatment of specific pain conditions, wherein the therapeutic molecule is a single chain, polypeptide fusion protein, comprising: a non-cytotoxic protease, or a fragment thereof, which protease or protease fragment is capable of cleaving a protein of the exocytic fusion apparatus of a nociceptive sensory afferent; a Targeting Moiety that is capable of binding to a Binding Site on the nociceptive sensory afferent, which Binding Site is capable of undergoing endocytosis to be incorporated into an endosome within the nociceptive sensory afferent; a protease cleavage site at which site the fusion protein is cleavable by a protease, wherein the protease cleavage site is located between the non-cytotoxic protease or fragment thereof and the Targeting Moiety; and a translocation domain that is capable of translocating the protease or protease fragment from within an endosome, across the endosomal membrane and into the cytosol of the nociceptive sensory afferent.

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Treatment of pain

This invention relates to the use of non-cytotoxic fusion proteins for the treatment of specific types of pain.

Toxins may be generally divided into two groups according to the type of effect that they have on a target cell. In more detail, the first group of toxins kill their natural target cells, and are therefore known as cytotoxic toxin molecules. This group of toxins is exemplified *inter alia* by plant toxins such as ricin, and abrin, and by bacterial toxins such as diphtheria toxin, and Pseudomonas exotoxin A. Cytotoxic toxins have attracted much interest in the design of "magic bullets" (e.g. immunoconjugates, which comprise a cytotoxic toxin component and an antibody that binds to a specific marker on a target cell) for the treatment of cellular disorders and conditions such as cancer. Cytotoxic toxins typically kill their target cells by inhibiting the cellular process of protein synthesis.

The second group of toxins, which are known as non-cytotoxic toxins, do not (as their name confirms) kill their natural target cells. Non-cytotoxic toxins have attracted much less commercial interest than have their cytotoxic counterparts, and exert their effects on a target cell by inhibiting cellular processes other than protein synthesis. Non-cytotoxic toxins are produced by a variety of plants, and by a variety of microorganisms such as Clostridium sp. and Neisseria sp.

Clostridial neurotoxins are proteins that typically have a molecular mass of the order of 150 kDa. They are produced by various species of bacteria, especially of the genus Clostridium, most importantly *C. tetani* and several strains of *C. botulinum*, *C. butyricum* and *C. argentinense*. There are at present eight different classes of the clostridial neurotoxin, namely: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C1, D, E, F and G, and they all share similar structures and modes of action.

Clostridial neurotoxins represent a major group of non-cytotoxic toxin molecules, and are synthesised by the host bacterium as single polypeptides that are modified post-translationally by a proteolytic cleavage event to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H-chain), which has a molecular mass of approximately 100 kDa, and the light chain (L-chain), which has a molecular mass of approximately 50 kDa.

L-chains possess a protease function (zinc-dependent endopeptidase activity) and exhibit a high substrate specificity for vesicle and/or plasma membrane associated proteins involved in the exocytic process. L-chains from different clostridial species or serotypes may hydrolyse different but specific peptide bonds in one of three substrate proteins, namely synaptobrevin, syntaxin or SNAP-25. These substrates are important components of the neurosecretory machinery.

Neisseria sp., most importantly from the species *N. gonorrhoeae*, produce functionally similar non-cytotoxic proteases. An example of such a protease is IgA protease (see WO99/58571).

It has been well documented in the art that toxin molecules may be re-targeted to a cell that is not the toxin's natural target cell. When so re-targeted, the modified toxin is capable of binding to a desired target cell and, following subsequent translocation into the cytosol, is capable of exerting its effect on the target cell. Said re-targeting is achieved by replacing the natural Targeting Moiety (TM) of the toxin with a different TM. In this regard, the TM is selected so that it will bind to a desired target cell, and allow subsequent passage of the modified toxin into an endosome within the target cell. The modified toxin also comprises a translocation domain to enable entry of the non-cytotoxic protease into the cell cytosol. The translocation domain can be the natural translocation domain of the toxin or it can be a different translocation domain obtained from a microbial protein with translocation activity.

The above-mentioned TM replacement may be effected by conventional chemical conjugation techniques, which are well known to a skilled person. In this regard, reference is made to Hermanson, G.T. (1996), Bioconjugate techniques, Academic Press, and to Wong, S.S. (1991), Chemistry of protein conjugation and cross-linking, CRC Press.

Chemical conjugation is, however, often imprecise. For example, following conjugation, a TM may become joined to the remainder of the conjugate at more than one attachment site.

Chemical conjugation is also difficult to control. For example, a TM may become joined to the remainder of the modified toxin at an attachment site on the protease component and/ or on the translocation component. This is problematic when attachment to only one of said components (preferably at a single site) is desired for therapeutic efficacy.

Thus, chemical conjugation results in a mixed population of modified toxin molecules, which is undesirable.

As an alternative to chemical conjugation, TM replacement may be effected by recombinant preparation of a single polypeptide fusion protein (see WO98/07864). This technique is based on the *in vivo* bacterial mechanism by which native clostridial neurotoxin (i.e. holotoxin) is prepared, and results in a fusion protein having the following structural arrangement:

NH_2 - [protease component] – [translocation component] – [TM] - COOH

According to WO98/07864, the TM is placed towards the C-terminal end of the fusion protein. The fusion protein is then activated by treatment with a protease, which cleaves at a site between the protease component and the translocation component. A di-chain protein is thus produced, comprising the protease component as a single polypeptide chain covalently attached (via a disulphide

bridge) to another single polypeptide chain containing the translocation component plus TM. Whilst the WO98/07864 methodology follows (in terms of structural arrangement of the fusion protein) the natural expression system of clostridial holotoxin, the present inventors have found that this system may result in the production of certain fusion proteins that have a substantially-reduced binding ability for the intended target cell.

This problem is particularly relevant in the context of treating specific types of pain.

The present invention addresses one or more of the above-mentioned problems by providing use of a therapeutic molecule for the manufacture of a medicament for the treatment of particular types of pain, wherein the therapeutic molecule is a single chain, polypeptide fusion protein, comprising:

a. a non-cytotoxic protease, or a fragment thereof, which protease or protease fragment is capable of cleaving a protein of the exocytic fusion apparatus in a nociceptive sensory afferent;

b. a Targeting Moiety that is capable of binding to a Binding Site on the nociceptive sensory afferent, which Binding Site is capable of undergoing endocytosis to be incorporated into an endosome within the nociceptive sensory afferent;

c. a protease cleavage site at which site the fusion protein is cleavable by a protease, wherein the protease cleavage site is located between the non-cytotoxic protease or fragment thereof and the Targeting Moiety; and

d. a translocation domain that is capable of translocating the protease or protease fragment from within an endosome, across the endosomal membrane and into the cytosol of the nociceptive sensory afferent.

The present inventors have found that the WO 98/07864 fusion protein system is not optimal for TMs requiring a N-terminal domain for interaction with a binding site on a nociceptive sensory afferent. This problem is particularly acute with TMs that require a specific N-terminus amino acid residue or a specific sequence of amino acid residues including the N-terminus amino acid residue for interaction with a binding site on a nociceptive sensory afferent.

In contrast to WO98/07864, the present invention employs non-cytotoxic fusion proteins, wherein the TM component of the fusion includes the relevant binding domain in an intra domain or an amino acid sequence located towards the middle (ie. of the linear peptide sequence) of the TM, or preferably located towards the N-terminus of the TM, or more preferably at or near to the N-terminus. The N-terminal domain is capable of binding to a Binding Site on a nociceptive sensory afferent, and the TM preferably has a requirement for a specific and defined sequence of amino acid residue(s) to be free at its N-terminus.

The compounds described here may be used to treat a patient suffering from one or more types of chronic pain including neuropathic pain, inflammatory pain, headache pain, somatic pain, visceral pain, and referred pain.

To "treat," as used here, means to deal with medically. It includes, for example, administering a compound of the invention to prevent pain or to lessen its severity.

The term "pain," as used here, means any unpleasant sensory experience, usually associated with a physical disorder. The physical disorder may or may not be apparent to a clinician. Pain is of two types: chronic and acute. An "acute pain" is a pain of short duration having a sudden onset. One type of acute pain, for example, is cutaneous pain felt on injury to the skin or other superficial tissues, such as caused by a cut or a burn. Cutaneous nociceptors terminate just below the skin, and due to the high concentration of nerve endings, produce a well-defined, localized pain of short duration. "Chronic pain" is a pain other than

an acute pain. Chronic pain includes neuropathic pain, inflammatory pain, headache pain, somatic pain visceral pain and referred pain.

I. Neuropathic Pain

The compounds of the invention may be used to treat pain caused by or otherwise associated with any of the following neuropathic pain conditions. "Neuropathic pain" means abnormal sensory input, resulting in discomfort, from the peripheral nervous system, central nervous systems, or both.

A. Symptoms of neuropathic pain

Symptoms of neuropathic pain can involve persistent, spontaneous pain, as well as allodynia (a painful response to a stimulus that normally is not painful), hyperalgesia (an accentuated response to a painful stimulus that usually causes only a mild discomfort, such as a pin prick), or hyperpathia (where a short discomfort becomes a prolonged severe pain).

B. Causes of neuropathic pain

Neuropathic pain may be caused by any of the following.

1. A traumatic insult, such as, for example, a nerve compression injury (e.g., a nerve crush, a nerve stretch, a nerve entrapment or an incomplete nerve transsection); a spinal cord injury (e.g., a hemisection of the spinal cord); a limb amputation; a contusion; an inflammation (e.g., an inflammation of the spinal cord); or a surgical procedure.

2. An ischemic event, including, for example, a stroke and heart attack.

3. An infectious agent

4. Exposure to a toxic agent, including, for example, a drug, an alcohol, a heavy metal (e.g., lead, arsenic, mercury), an industrial agent (e.g., a solvent, fumes from a glue) or nitrous oxide.

5. A disease, including, for example, an inflammatory disorder, a neoplastic tumor, an acquired immune deficiency syndrome (AIDS), Lymes disease, a leprosy, a metabolic disease, a peripheral nerve disorder, like neuroma, a mononeuropathy or a polyneuropathy.

C. Types of neuropathic pain

1. Neuralgia.

A neuralgia is a pain that radiates along the course of one or more specific nerves usually without any demonstrable pathological change in the nerve structure. The causes of neuralgia are varied. Chemical irritation, inflammation, trauma (including surgery), compression by nearby structures (for instance, tumors), and infections may all lead to neuralgia. In many cases, however, the cause is unknown or unidentifiable. Neuralgia is most common in elderly persons, but it may occur at any age. A neuralgia, includes, without limitation, a trigeminal neuralgia, a post-herpetic neuralgia, a postherpetic neuralgia, a glossopharyngeal neuralgia, a sciatica and an atypical facial pain.

Neuralgia is pain in the distribution of a nerve or nerves. Examples are trigeminal neuralgia, atypical facial pain, and postherpetic neuralgia (caused by shingles or herpes). The affected nerves are responsible for sensing touch, temperature and pressure in the facial area from the jaw to the forehead. The disorder generally causes short episodes of excruciating pain, usually for less than two minutes and on only one side of the face. The pain can be described in a variety of ways such as "stabbing," "sharp," "like lightning," "burning," and even "itchy". In the atypical form of TN, the pain can also present as severe or merely aching and last for extended periods. The pain associated with TN is recognized as one the most excruciating pains that can be experienced.

Simple stimuli such as eating, talking, washing the face, or any light touch or sensation can trigger an attack (even the sensation of a gentle breeze). The attacks can occur in clusters or as an isolated attack.

Symptoms include sharp, stabbing pain or constant, burning pain located anywhere, usually on or near the surface of the body, in the same location for each episode; pain along the path of a specific nerve; impaired function of affected body part due to pain, or muscle weakness due to concomitant motor

nerve damage; increased sensitivity of the skin or numbness of the affected skin area (feeling similar to a local anesthetic such as a Novacaine shot); and any touch or pressure is interpreted as pain. Movement may also be painful.

Trigeminal neuralgia is the most common form of neuralgia. It affects the main sensory nerve of the face, the trigeminal nerve ("trigeminal" literally means "three origins", referring to the division of the nerve into 3 branches). This condition involves sudden and short attacks of severe pain on the side of the face, along the area supplied by the trigeminal nerve on that side. The pain attacks may be severe enough to cause a facial grimace, which is classically referred to as a painful tic (tic douloureux). Sometimes, the cause of trigeminal neuralgia is a blood vessel or small tumor pressing on the nerve. Disorders such as multiple sclerosis (an inflammatory disease affecting the brain and spinal cord), certain forms of arthritis, and diabetes (high blood sugar) may also cause trigeminal neuralgia, but a cause is not always identified. In this condition, certain movements such as chewing, talking, swallowing, or touching an area of the face may trigger a spasm of excruciating pain.

A related but rather uncommon neuralgia affects the glosso-pharyngeal nerve, which provides sensation to the throat. Symptoms of this neuralgia are short, shock-like episodes of pain located in the throat.

Neuralgia may occur after infections such as shingles, which is caused by the varicella-zoster virus, a type of herpesvirus. This neuralgia produces a constant burning pain after the shingles rash has healed. The pain is worsened by movement of or contact with the affected area. Not all of those diagnosed with shingles go on to experience postherpetic neuralgia, which can be more painful than shingles. The pain and sensitivity can last for months or even years. The pain is usually in the form of an intolerable sensitivity to any touch but especially light touch. Postherpetic neuralgia is not restricted to the face; it can occur anywhere on the body but usually occurs at the location of the shingles rash.

Depression is not uncommon due to the pain and social isolation during the illness.

Postherpetic neuralgia may be debilitating long after signs of the original herpes infection have disappeared. Other infectious diseases that may cause neuralgia are syphilis and Lyme disease.

Diabetes is another common cause of neuralgia. This very common medical problem affects almost 1 out of every 20 Americans during adulthood. Diabetes damages the tiny arteries that supply circulation to the nerves, resulting in nerve fiber malfunction and sometimes nerve loss. Diabetes can produce almost any neuralgia, including trigeminal neuralgia, carpal tunnel syndrome (pain and numbness of the hand and wrist), and meralgia paresthetica (numbness and pain in the thigh due to damage to the lateral femoral cutaneous nerve). Strict control of blood sugar may prevent diabetic nerve damage and may accelerate recovery in patients who do develop neuralgia.

Other medical conditions that may be associated with neuralgias are chronic renal insufficiency and porphyria -- a hereditary disease in which the body cannot rid itself of certain substances produced after the normal breakdown of blood in the body. Certain drugs may also cause this problem.

2. Deafferentation.

Deafferentation indicates a loss of the sensory input from a portion of the body, and can be caused by interruption of either peripheral sensory fibres or nerves from the central nervous system. A deafferentation pain syndrome, includes, without limitation, an injury to the brain or spinal cord, a post-stroke pain, a phantom pain, a paraplegia, a brachial plexus avulsion injuries, lumbar radiculopathies.

3. Complex regional pain syndromes (CRPSs)

CRPS is a chronic pain syndrome resulting from sympathetically-maintained pain, and presents in two forms. CRPS 1 currently replaces the term "reflex sympathetic dystrophy syndrome". It is a chronic nerve disorder that occurs most often in the arms or legs after a minor or major injury. CRPS 1 is associated with severe pain; changes in the nails, bone, and skin; and an increased sensitivity to touch in the affected limb. CRPS 2 replaces the term causalgia, and results from an identified injury to the nerve. A CRPS, includes, without limitation, a CRPS Type I (reflex sympathetic dystrophy) and a CRPS Type II (causalgia).

4. Neuropathy.

A neuropathy is a functional or pathological change in a nerve and is characterized clinically by sensory or motor neuron abnormalities.

Central neuropathy is a functional or pathological change in the central nervous system.

Peripheral neuropathy is a functional or pathological change in one or more peripheral nerves. The peripheral nerves relay information from your central nervous system (brain and spinal cord) to muscles and other organs and from your skin, joints, and other organs back to your brain. Peripheral neuropathy occurs when these nerves fail to carry information to and from the brain and spinal cord, resulting in pain, loss of sensation, or inability to control muscles. In some cases, the failure of nerves that control blood vessels, intestines, and other organs results in abnormal blood pressure, digestion problems, and loss of other basic body processes. Risk factors for neuropathy include diabetes, heavy alcohol use, and exposure to certain chemicals and drugs. Some people have a hereditary predisposition for neuropathy. Prolonged pressure on a nerve is another risk for developing a nerve injury. Pressure injury may be caused by prolonged immobility (such as a long surgical procedure or lengthy illness) or compression of a nerve by casts, splints, braces, crutches, or other devices. Polyneuropathy implies a widespread process that usually affects both sides of the body equally. The symptoms depend on which type of nerve is affected. The

three main types of nerves are sensory, motor, and autonomic. Neuropathy can affect any one or a combination of all three types of nerves. Symptoms also depend on whether the condition affects the whole body or just one nerve (as from an injury). The cause of chronic inflammatory polyneuropathy is an abnormal immune response. The specific antigens, immune processes, and triggering factors are variable and in many cases are unknown. It may occur in association with other conditions such as HIV, inflammatory bowel disease, lupus erythematosis, chronic active hepatitis, and blood cell abnormalities.

Peripheral neuropathy may involve a function or pathological change to a single nerve or nerve group (monneuropathy) or a function or pathological change affecting multiple nerves (polyneuropathy).

Peripheral neuropathies

Hereditary disorders

- Charcot-Marie-Tooth disease
- Friedreich's ataxia

Systemic or metabolic disorders

- Diabetes (diabetic neuropathy)
- Dietary deficiencies (especially vitamin B-12)
- Excessive alcohol use (alcoholic neuropathy)
- Uremia (from kidney failure)
- Cancer

Infectious or inflammatory conditions

- AIDS
- Hepatitis
- Colorado tick fever
- diphtheria
- Guillain-Barre syndrome
- HIV infection without development of AIDS
- leprosy
- Lyme

polyarteritis nodosa
rheumatoid arthritis
sarcoidosis
Sjogren syndrome
syphilis
systemic lupus erythematosus
amyloid

Exposure to toxic compounds

sniffing glue or other toxic compounds
nitrous oxide
industrial agents -- especially solvents
heavy metals (lead, arsenic, mercury, etc.)
Neuropathy secondary to drugs like analgesic nephropathy

Miscellaneous causes

ischemia (decreased oxygen/decreased blood flow)
prolonged exposure to cold temperature

a. Polyneuropathy

Polyneuropathy is a peripheral neuropathy involving the loss of movement or sensation to an area caused by damage or destruction to multiple peripheral nerves. Polyneuropathic pain, includes, without limitation, post-polio syndrome, postmastectomy syndrome, diabetic neuropathy, alcohol neuropathy, amyloid, toxins, AIDS, hypothyroidism, uremia, vitamin deficiencies, chemotherapy-induced pain, 2',3'-didexoycytidine (ddC) treatment, Guillain-Barré syndrome or Fabry's disease.

b. Mononeuropathy

Mononeuropathy is a peripheral neuropathy involving loss of movement or sensation to an area caused by damage or destruction to a single peripheral nerve or nerve group. Mononeuropathy is most often caused by damage to a local area resulting from injury or trauma, although occasionally systemic disorders may cause isolated nerve damage (as with mononeuritis multiplex). The usual causes are direct trauma, prolonged pressure on the nerve, and compression of the nerve by swelling or injury to nearby body structures. The

damage includes destruction of the myelin sheath (covering) of the nerve or of part of the nerve cell (the axon). This damage slows or prevents conduction of impulses through the nerve. Mononeuropathy may involve any part of the body. Mononeuropathic pain, includes, without limitation, a sciatic nerve dysfunction, a common peroneal nerve dysfunction. a radial nerve dysfunction, an ulnar nerve dysfunction, a cranial mononeuropathy VI, a cranial mononeuropathy VII, a cranial mononeuropathy III (compression type), a cranial mononeuropathy III (diabetic type), an axillary nerve dysfunction, a carpal tunnel syndrome, a femoral nerve dysfunction, a tibial nerve dysfunction, a Bell's palsy, a thoracic outlet syndrome, a carpal tunnel syndrome and a sixth (abducent) nerve palsy

c. Generalized peripheral neuropathies

Generalized peripheral neuropathis are symmetrical, and usually due to various systematic illnesses and disease processes that affect the peripheral nervous system in its entirety. They are further subdivided into several categories:

i. Distal axonopathies are the result of some metabolic or toxic derangement of neurons. They may be caused by metabolic diseases such as diabetes, renal failure, deficiency syndromes such as malnutrition and alcoholism, or the effects of toxins or drugs. Distal axonopathy (aka dying back neuropathy) is a type of peripheral neuropathy that results from some metabolic or toxic derangement of peripheral nervous system (PNS) neurons. It is the most common response of nerves to metabolic or toxic disturbances, and as such may be caused by metabolic diseases such as diabetes, renal failure, deficiency syndromes such as malnutrition and alcoholism, or the effects of toxins or drugs. The most common cause of distal axonopathy is diabetes, and the most common distal axonopathy is diabetic neuropathy.

ii. Myelinopathies are due to a primary attack on myelin causing an acute failure of impulse conduction. The most common cause is acute inflammatory demyelinating polyneuropathy (AIDP; aka Guillain-Barré syndrome), though other causes include chronic inflammatory demyelinating syndrome (CIDP), genetic metabolic disorders (e.g., leukodystrophy), or toxins. Myelinopathy is due to primary destruction of myelin or the myelinating Schwann

cells, which leaves the axon intact, but causes an acute failure of impulse conduction. This demyelination slows down or completely blocks the conduction of electical impulses through the nerve. The most common cause is acute inflammatory demyelinating polyneuropathy (AIDP, better known as Guillain-Barré syndrome), though other causes include chronic inflammatory demyelinating polyneuropathy (CIDP), genetic metabolic disorders (e.g., leukodystrophy or Charcot-Marie-Tooth disease), or toxins.

iii. Neuronopathies are the result of destruction of peripheral nervous system (PNS) neurons. They may be caused by motor neurone diseases, sensory neuronopathies (e.g., Herpes zoster), toxins or autonomic dysfunction. Neurotoxins may cause neuronopathies, such as the chemotherapy agent vincristine. Neuronopathy is dysfunction due to damage to neurons of the peripheral nervous system (PNS), resulting in a peripheral neuropathy. It may be caused by motor neurone diseases, sensory neuronopathies (e.g., Herpes zoster), toxic substances or autonomic dysfunction. A person with neuronopathy may present in different ways, depending on the cause, the way it affects the nerve cells, and the type of nerve cell that is most affected.

iv. Focal entrapment neuropathies (e.g., carpal tunnel syndrome).

II. Inflammatory pain

The compounds of the invention may be used to treat pain caused by or otherwise associated with any of the following inflammatory conditions

A. Arthritic disorder

Arthritic disorders include, for example, a rheumatoid arthritis; a juvenile rheumatoid arthritis; a systemic lupus erythematosus (SLE); a gouty arthritis; a scleroderma; an osteoarthritis; a psoriatic arthritis; an ankylosing spondylitis; a Reiter's syndrome (reactive arthritis); an adult Still's disease; an arthritis from a viral infection; an arthritis from a bacterial infection, such as, e.g., a gonococcal arthritis and a non-gonococcal bacterial arthritis (septic arthritis); a Tertiary Lyme disease; a tuberculous arthritis; and an arthritis from a fungal infection, such as, e.g., a blastomycosis

B. Autoimmune diseases

Autoimmune diseases include, for example, a Guillain-Barré syndrome, a Hashimoto's thyroiditis, a pernicious anemia, an Addison's disease, a type I diabetes, a systemic lupus erythematosus, a dermatomyositis, a Sjogren's syndrome, a lupus erythematosus, a multiple sclerosis, a myasthenia gravis, a Reiter's syndrome and a Grave's disease.

C. Connective tissue disorder

Connective tissue disorders include, for example, a spondyloarthritis a dermatomyositis, and a fibromyalgia.

D. Injury

Inflammation caused by injury, including, for example, a crush, puncture, stretch of a tissue or joint, may cause chronic inflammatory pain.

E. Infection

Inflammation caused by infection, including, for example, a tuberculosis or an interstitial keratitis may cause chronic inflammatory pain.

F. Neuritis

Neuritis is an inflammatory process affecting a nerve or group of nerves. Symptoms depend on the nerves involved, but may include pain, paresthesias, paresis, or hypesthesia (numbness).

Examples include:

a. Brachial neuritis

b. Retrobulbar neuropathy, an inflammatory process affecting the part of the optic nerve lying immediately behind the eyeball.

c. Optic neuropathy, an inflammatory process affecting the optic nerve causing sudden, reduced vision in the affected eye. The cause of optic neuritis is unknown. The sudden inflammation of the optic nerve (the nerve connecting the eye and the brain) leads to swelling and destruction of the myelin sheath. The inflammation may occasionally be the result of a viral infection, or it may be

caused by autoimmune diseases such as multiple sclerosis. Risk factors are related to the possible causes.

d. Vestibular neuritis, a viral infection causing an inflammatory process affecting the vestibular nerve.

G. Joint inflammation

Inflammation of the joint, such as that caused by bursitis or tendonitis, for example, may cause chronic inflammatory pain.

III. Headache Pain

The compounds of the invention may be used to treat pain caused by or otherwise associated with any of the following headache conditions. A headache (medically known as cephalgia) is a condition of mild to severe pain in the head; sometimes neck or upper back pain may also be interpreted as a headache. It may indicate an underlying local or systemic disease or be a disorder in itself.

A. Muscular/myogenic headache

Muscular/myogenic headaches appear to involve the tightening or tensing of facial and neck muscles; they may radiate to the forehead. Tension headache is the most common form of myogenic headache.

A tension headache is a condition involving pain or discomfort in the head, scalp, or neck, usually associated with muscle tightness in these areas. Tension headaches result from the contraction of neck and scalp muscles. One cause of this muscle contraction is a response to stress, depression or anxiety. Any activity that causes the head to be held in one position for a long time without moving can cause a headache. Such activities include typing or use of computers, fine work with the hands, and use of a microscope. Sleeping in a cold room or sleeping with the neck in an abnormal position may also trigger this type of headache. A tension-type headache, includes, without limitation, an episodic tension headache and a chronic tension headache.

B. Vascular headache

The most common type of vascular headache is migraine. Other kinds of vascular headaches include cluster headaches, which cause repeated episodes of intense pain, and headaches resulting from high blood pressure

1. Migraine

A migraine is a heterogeneous disorder that generally involves recurring headaches. Migraines are different from other headaches because they occur with other symptoms, such as, e.g., nausea, vomiting, or sensitivity to light. In most people, a throbbing pain is felt only on one side of the head. Clinical features such as type of aura symptoms, presence of prodromes, or associated symptoms such as vertigo, may be seen in subgroups of patients with different underlying pathophysiological and genetic mechanisms. A migraine headache, includes, without limitation, a migraine without aura (common migraine), a migraine with aura (classic migraine), a menstrual migraine, a migraine equivalent (acephalic headache), a complicated migraine, an abdominal migraine and a mixed tension migraine.

2. Cluster headache

Cluster headaches affect one side of the head (unilateral) and may be associated with tearing of the eyes and nasal congestion. They occurs in clusters, happening repeatedly every day at the same time for several weeks and then remitting.

D. High blood pressure headache

E. Traction and inflammatory headache

Traction and inflammatory headaches are usually symptoms of other disorders, ranging from stroke to sinus infection.

F. Hormone headache

G. Rebound headache

Rebound headaches, also known as medication overuse headaches, occur when medication is taken too frequently to relieve headache. Rebound headaches frequently occur daily and can be very painful.

H. Chronic sinusitis headache

Sinusitis is inflammation, either bacterial, fungal, viral, allergic or autoimmune, of the paranasal sinuses. Chronic sinusitis is one of the most common complications of the common cold. Symptoms include: Nasal congestion; facial pain; headache; fever; general malaise; thick green or yellow discharge; feeling of facial 'fullness' worsening on bending over. In a small number of cases, chronic maxillary sinusitis can also be brought on by the spreading of bacteria from a dental infection. Chronic hyperplastic eosinophilic sinusitis is a noninfective form of chronic sinusitis.

I. An organic headache

J. Ictal headaches

Ital headaches are headaches associated with seizure activity.

IV. Somatic pain

The compounds of the invention may be used to treat pain caused by or otherwise associated with any of the following somatic pain conditions. Somatic pain originates from ligaments, tendons, bones, blood vessels, and even nerves themselves. It is detected with somatic nociceptors. The scarcity of pain receptors in these areas produces a dull, poorly-localized pain of longer duration than cutaneous pain; examples include sprains and broken bones. Additional examples include the following.

A. Excessive muscle tension

Excessive muclse tension can be caused, for example, by a sprain or a strain.

B. Repetitive motion disorders

Repetitive motion disorders can result from overuse of the hands, wrists, elbows, shoulders, neck, back, hips, knees, feet, legs, or ankles.

C. Muscle disorders

Muscle disorders causing somatic pain include, for example, a polymyositis, a dermatomyositis, a lupus, a fibromyalgia, a polymyalgia rheumatica, and a rhabdomyolysis.

D. Myalgia

Myalgia is muscle pain and is a symptom of many diseases and disorders. The most common cause for myalgia is either overuse or over-stretching of a muscle or group of muscles. Myalgia without a traumatic history is often due to viral infections. Longer-term myalgias may be indicative of a metabolic myopathy, some nutritional deficiencies or chronic fatigue syndrome.

E. Infection

Infection can cause somatic pain. Examples of such infection include, for example, an abscess in the muscle, a trichinosis, an influenza, a Lyme disease, a malaria, a Rocky Mountain spotted fever, Avian influenza, the common cold, community-acquired pneumonia, meningitis, monkeypox, Severe Acute Respiratory Syndrome, toxic shock syndrome, trichinosis, typhoid fever, and upper respiratory tract infection.

F. Drugs

Drugs can cause somatic pain. Such drugs include, for example, cocaine, a statin for lowering cholesterol (such as atorvastatin, simvastatin, and lovastatin), and an ACE inhibitor for lowering blood pressure (such as enalapril and captopril)

V. Visceral pain

The compounds of the invention may be used to treat pain caused by or otherwise associated with any of the following visceral pain conditions. Visceral pain originates from body's viscera, or organs. Visceral nociceptors are located

within body organs and internal cavities. The even greater scarcity of nociceptors in these areas produces pain that is usually more aching and of a longer duration than somatic pain. Visceral pain is extremely difficult to localise, and several injuries to visceral tissue exhibit "referred" pain, where the sensation is localised to an area completely unrelated to the site of injury. Examples of visceral pain include the following.

A. Functional visceral pain

Functional visceral pain includes, for example, an irritable bowel syndrome and a chronic functional abdominal pain (CFAP), a functional constipation and a functional dyspepsia, a non-cardiac chest pain (NCCP) and a chronic abdominal pain.

B. Chronic gastrointestinal inflammation

Chronic gastrointestinal inflammation includes, for example, a gastritis, an inflammatory bowel disease, like, e.g., a Crohn's disease, an ulcerative colitis, a microscopic colitis, a diverticulitis and a gastroenteritis; an interstitial cystitis; an intestinal ischemia; a cholecystitis; an appendicitis; a gastroesophageal reflux; an ulcer, a nephrolithiasis, an urinary tract infection, a pancreatitis and a hernia.

C. Autoimmune pain

Autoimmune pain includes, for example, a sarcoidosis and a vasculitis.

D. Orangic visceral pain

Organic visceral pain includes, for example, pain resulting from a traumatic, inflammatory or degenerative lesion of the gut or produced by a tumor impinging on sensory innervation.

E. Treatment-induced visceral pain

Treatment-induced visceral pain includes, for example, a pain attendant to chemotherapy therapy or a pain attendant to radiation therapy.

VI. Referred pain

The compounds of the invention may be used to treat pain caused by or otherwise associated with any of the following referred pain conditions.

Referred pain arises from pain localized to an area separate from the site of pain stimulation. Often, referred pain arises when a nerve is compressed or damaged at or near its origin. In this circumstance, the sensation of pain will generally be felt in the territory that the nerve serves, even though the damage originates elsewhere. A common example occurs in intervertebral disc herniation, in which a nerve root arising from the spinal cord is compressed by adjacent disc material. Although pain may arise from the damaged disc itself, pain will also be felt in the region served by the compressed nerve (for example, the thigh, knee, or foot). Relieving the pressure on the nerve root may ameliorate the referred pain, provided that permanent nerve damage has not occurred. Myocardial ischaemia (the loss of blood flow to a part of the heart muscle tissue) is possibly the best known example of referred pain; the sensation can occur in the upper chest as a restricted feeling, or as an ache in the left shoulder, arm or even hand.

The non-cytotoxic protease component of the present invention is a non-cytotoxic protease, or a fragment thereof, which protease or protease fragment is capable of cleaving different but specific peptide bonds in one of three substrate proteins, namely synaptobrevin, syntaxin or SNAP-25, of the exocytic fusion apparatus in a nociceptive sensory afferent. These substrates are important components of the neurosecretory machinery. The non-cytotoxic protease component of the present invention is preferably a neisserial IgA protease or a fragment thereof or a clostridial neurotoxin L-chain or a fragment thereof. A particularly preferred non-cytotoxic protease component is a botulinum neurotoxin (BoNT) L-chain or a fragment thereof.

The translocation component of the present invention enables translocation of the non-cytotoxic protease (or fragment thereof) into the target cell such that

functional expression of protease activity occurs within the cytosol of the target cell. The translocation component is preferably capable of forming ion-permeable pores in lipid membranes under conditions of low pH. Preferably it has been found to use only those portions of the protein molecule capable of pore-formation within the endosomal membrane. The translocation component may be obtained from a microbial protein source, in particular from a bacterial or viral protein source. Hence, in one embodiment, the translocation component is a translocating domain of an enzyme, such as a bacterial toxin or viral protein. The translocation component of the present invention is preferably a clostridial neurotoxin H-chain or a fragment thereof. Most preferably it is the H_N domain (or a functional component thereof), wherein H_N means a portion or fragment of the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain.

The TM component of the present invention is responsible for binding the fusion protein of the present invention to a Binding Site on a target cell. Thus, the TM component is simply a ligand through which a fusion protein of the present invention binds to a selected target cell.

In the context of the present invention, the target cell is a nociceptive sensory afferent, preferably a primary nociceptive afferent (e.g. an A-fibre such as an Aδ-fibre or a C-fibre). Thus, the fusion proteins of the present invention are capable of inhibiting neurotransmitter or neuromodulator [e.g. glutamate, substance P, calcitonin-gene related peptide (CGRP), and/ or neuropeptide Y] release from discrete populations of nociceptive sensory afferent neurons. In use, the fusion proteins reduce or prevent the transmission of sensory afferent signals (e.g. neurotransmitters or neuromodulators) from peripheral to central pain fibres, and therefore have application as therapeutic molecules for the treatment of pain, in particular chronic pain.

It is routine to confirm that a TM binds to a nociceptive sensory afferent. For example, a simple radioactive displacement experiment may be employed in which tissue or cells representative of the nociceptive sensory afferent (for example DRGs) are exposed to labelled (e.g. tritiated) ligand in the presence of an excess of unlabelled ligand. In such an experiment, the relative proportions of non-specific and specific binding may be assessed, thereby allowing confirmation that the ligand binds to the nociceptive sensory afferent target cell. Optionally, the assay may include one or more binding antagonists, and the assay may further comprise observing a loss of ligand binding. Examples of this type of experiment can be found in Hulme, E.C. (1990), Receptor-binding studies, a brief outline, pp. 303-311, In Receptor biochemistry, A Practical Approach, Ed. E.C. Hulme, Oxford University Press.

The fusion proteins of the present invention generally demonstrate a reduced binding affinity (in the region of up to 100-fold) for nociceptive sensory afferent target cells when compared with the corresponding 'free' TM. However, despite this observation, the fusion proteins of the present invention surprisingly demonstrate good efficacy. This can be attributed to two principal features. First, the non-cytotoxic protease component is catalytic – thus, the therapeutic effect of a few such molecules is rapidly amplified. Secondly, the receptors present on the nociceptive sensory afferents need only act as a gateway for entry of the therapeutic, and need not necessarily be stimulated to a level required in order to achieve a ligand-receptor mediated pharmacological response. Accordingly, the fusion proteins of the present invention may be administered at a dosage that is much lower that would be employed for other types of analgesic molecules such as NSAIDS, morphine, and gabapentin. The latter molecules are typically administered at high microgram to milligram (even up to hundreds of milligram) quantities, whereas the fusion proteins of the present invention may be administered at much lower dosages, typically at least 10-fold lower, and more typically at 100-fold lower.

The TM preferably comprises a maximum of 50 amino acid residues, more preferably a maximum of 40 amino acid residues, particularly preferably a maximum of 30 amino acid residues, and most preferably a maximum of 20 amino acid residues.

Opioids represent a preferred group of TMs of the present invention. Within this family of peptides is included enkephalins (met and leu), endomorphins 1 and 2, β-endorphin and dynorphin. Opioid peptides are frequently used in the clinic to modify the activity to nociceptors, and other cells involved in the pain response. As exemplified by the three-step World Health Organisation Analgesic Ladder, opioids have entry points into the pharmacological treatment of chronic cancer and non-cancer pain at all three stages, underlining their importance to the treatment of pain. Reference to opioids embraces fragments, variants and derivatives thereof, which retain the ability to bind to nociceptive sensory afferents.

The TM of the invention can also be a molecule that acts as an "agonist" at one or more of the receptors present on a nociceptive sensory afferent, more particularly on a primary nociceptive afferent. Conventionally, an agonist has been considered any molecule that can either increase or decrease activities within a cell, namely any molecule that simply causes an alteration of cell activity. For example, the conventional meaning of an agonist would include a chemical substance capable of combining with a receptor on a cell and initiating a reaction or activity, or a drug that induces an active response by activating receptors, whether the response is an increase or decrease in cellular activity.

However, for the purposes of this invention, an agonist is more specifically defined as a molecule that is capable of stimulating the process of exocytic fusion in a target cell, which process is susceptible to inhibition by a protease (or fragment thereof) capable of cleaving a protein of the exocytic fusion apparatus in said target cell.

Accordingly, the particular agonist definition of the present invention would exclude many molecules that would be conventionally considered as agonists. For example, nerve growth factor (NGF) is an agonist in respect of its ability to promote neuronal differentiation via binding to a TrkA receptor. However, NGF is not an agonist when assessed by the above criteria because it is not a principal inducer of exocytic fusion. In addition, the process that NGF stimulates (i.e. cell differentiation) is not susceptible to inhibition by the protease activity of a non-cytotoxic toxin molecule.

The agonist properties of a TM that binds to a receptor on a nociceptive afferent can be confirmed using the methods described in Example 10.

In a preferred embodiment of the invention, the target for the TM is the ORL_1 receptor. This receptor is a member of the G-protein-coupled class of receptors, and has a seven transmembrane domain structure. The properties of the ORL_1 receptor are discussed in detail in Mogil & Pasternak (2001), *Pharmacological Reviews,* Vol. 53, No. 3, pages 381-415.

In one embodiment, the TM is a molecule that binds (preferably that specifically binds) to the ORL_1 receptor. More preferably, the TM is an "agonist" of the ORL_1 receptor. The term "agonist" in this context is defined as above.

The agonist properties of a TM that binds to an ORL_1 receptor can be confirmed using the methods described in Example 10. These methods are based on previous experiments [see Inoue *et al.* 1998 [Proc. Natl. Acad. Sci., 95, 10949-10953]), which confirm that the natural agonist of the ORL_1 receptor, nociceptin, causes the induction of substance P release from nociceptive primary afferent neurons. This is supported by the fact that:

- the nociceptin-induced responses are abolished by specific NK1 receptor (the substance P receptor) antagonists; and

- pre-treatment of the cells with capsaicin (which depletes substance P from small diameter primary afferent neurons) attenuates the nociceptin-induced responses.

Similarly, Inoue *et al.* confirm that an intraplantar injection of botulinum neurotoxin type A abolishes the nociceptin-induced responses. Since it is known that BoNT inhibits the release of substance P from primary afferent neurons (Welch et al., 2000, Toxicon, 38, 245-258), this confirms the link between nociceptin-ORL_1 interaction and subsequent release of substance P.

Thus, a TM can be said to have agonist activity at the ORL_1 receptor if the TM causes an induction in the release of substance P from a nociceptive sensory afferent neuron (see Example 10).

In a particularly preferred embodiment of the invention, the TM is nociceptin - the natural ligand for the ORL_1 receptor. Nociceptin targets the ORL_1 receptor with high affinity. Examples of other preferred TMs include:

Code	Sequence	Ref.	SEQ ID No.
Nociceptin 1-17	FGGFTGARKSARKLANQ	[1]	37,38
Nociceptin 1-11	FGGFTGARKSA	[1]	39,40
Nociceptin [Y10]1-11	FGGFTGARKYA	[1]	41,42
Nociceptin [Y11]1-11	FGGFTGARKSY	[1]	43,44
Nociceptin [Y14]1-17	FGGFTGARKSARKYANQ	[1]	45,46
Nociceptin 1-13	FGGFTGARKSARK	[2]	47,48

Code	Sequence	Ref.	SEQ ID No.
Nociceptin [R14K15] 1-17 (also known in this specification as "variant" nociceptin)	FGGFTGARKSARKRKNQ	[3,4]	49,50
Peptide agonist	Peptide agonists from combinatorial library approach	[5]	-

[1] Mogil & Pasternak, 2001, Pharmacol. Rev., 53, 381-415

[2] Maile *et al.*, 2003, Neurosci. Lett., 350, 190-192

[3] Rizzi *et al.*, 2002, J. Pharmacol. Exp. Therap., 300, 57-63

[4] Okada *et al.*, 2000, Biochem. Biophys. Res. Commun., 278, 493-498

[5] Dooley *et al.*, 1997, J Pharmacol Exp Ther. 283(2), 735-41.

The above-identified "variant" TM demonstrates particularly good binding affinity (when compared with natural nociceptin) for nociceptive sensory afferents. This is surprising as the amino acid modifications occur at a position away from the N-terminus of the TM. Moreover, the modifications are almost at the C-terminus of the TM, which in turn is attached to a large polypeptide sequence (i.e. the translocation domain). Generally speaking, a TM-containing fusion protein will demonstrate an approximate 100-fold reduction in binding ability *vis-à-vis* the TM *per se*. The above-mentioned "variant" TM *per se* demonstrates an approximate 3- to 10-fold increase in binding ability for a nociceptive sensory afferent (e.g. via the ORL1 receptor) *vis-à-vis* natural nociceptin. Thus, a "variant" TM-containing fusion might be expected to demonstrate an approximate 10-fold reduction in binding ability for a nociceptive sensory afferent (e.g. via the ORL1 receptor) *vis-à-vis* 'free' nociceptin. However, the present inventors have demonstrated that such "variant" TM-containing fusion proteins demonstrate a binding ability that (most surprisingly) closely mirrors that of 'free' nociceptin – see Figure 14.

In the context of the present invention, the term opioid or an agonist of the ORL_1 receptor (such as nociceptin, or any one of the peptides listed in the table above) embraces molecules having at least 70%, preferably at least 80%, more preferably at least 90%, and most preferably at least 95% homology with said opioid or agonist. The agonist homologues retain the agonist properties of nociceptin at the ORL_1 receptor, which may be tested using the methods provided in Example 10. Similarly, an opioid homologue substantially retains the binding function of the opioid with which it shows high homology.

The invention also encompasses fragments, variants, and derivatives of any one of the TMs described above. These fragments, variants, and derivatives substantially retain the properties that are ascribed to said TMs.

In addition to the above-mentioned opioid and non-opioid classes of TMs, a variety of other polypeptides are suitable for targeting the fusion proteins of the present invention to nociceptive sensory afferents (e.g. to nociceptors). In this regard, particular reference is made to galanin and derivatives of galanin. Galanin receptors are found pre- and post-synaptically in DRGs (Liu & Hokfelt, (2002), Trends Pharm. Sci., 23(10), 468-74), and are enhanced in expression during neuropathic pain states. Proteinase-activated receptors (PARs) are also a preferred group of TMs of the present invention, most particularly PAR-2. It is known that agonists of PAR-2 induce/ elicit acute inflammation, in part via a neurogenic mechanism. PAR2 is expressed by primary spinal afferent neurons, and PAR2 agonists stimulate release of substance P (SP) and calcitonin gene-related peptide (CGRP) in peripheral tissues

A particularly preferred set of TMs of the present invention includes:

Ligand	Reference
Nociceptin	**Guerrini, *et al.***, (1997) J. Med. Chem., 40, pp. 1789-1793

Ligand	Reference
β-endorphin	**Blanc, *et al.*,** (1983) J. Biol. Chem., 258(13), pp. 8277-8284
Endomorphin-1; Endomorphin-2	**Zadina, *et al.*,** (1997). Nature, 386, pp. 499-502
Dynorphin	**Fields & Basbaum** (2002) Chapter 11, In The Textbook of Pain, Wall & Melzack eds.
Met-enkephalin	**Fields & Basbaum** (2002) Chapter 11, In The Textbook of Pain, Wall & Melzack eds.
Leu-enkephalin	**Fields & Basbaum** (2002) Chapter 11, In The Textbook of Pain, Wall & Melzack eds.
Galanin	**Xu *et al.*,** (2000) Neuropeptides, **34** (3&4), 137–147
PAR-2 peptide	**Vergnolle *et al.***, (2001) Nat. Med., 7(7), 821-826

The protease cleavage site of the present invention allows cleavage (preferably controlled cleavage) of the fusion protein at a position between the non-cytotoxic protease component and the TM component. It is this cleavage reaction that converts the fusion protein from a single chain polypeptide into a disulphide-linked, di-chain polypeptide.

According to a preferred embodiment of the present invention, the TM binds via a domain or amino acid sequence that is located away from the C-terminus of the TM. For example, the relevant binding domain may include an intra domain or an amino acid sequence located towards the middle (i.e. of the linear peptide sequence) of the TM. Preferably, the relevant binding domain is located towards the N-terminus of the TM, more preferably at or near to the N-terminus.

In one embodiment, the single chain polypeptide fusion may include more than one proteolytic cleavage site. However, where two or more such sites exist, they are different, thereby substantially preventing the occurrence of multiple cleavage events in the presence of a single protease. In another embodiment, it is preferred that the single chain polypeptide fusion has a single protease cleavage site.

The protease cleavage sequence(s) may be introduced (and/ or any inherent cleavage sequence removed) at the DNA level by conventional means, such as by site-directed mutagenesis. Screening to confirm the presence of cleavage sequences may be performed manually or with the assistance of computer software (e.g. the MapDraw program by DNASTAR, Inc.).

Whilst any protease cleavage site may be employed, the following are preferred:

Enterokinase	(DDDDK↓)
Factor Xa	(IEGR↓ / IDGR↓)
TEV(Tobacco Etch virus)	(ENLYFQ↓G)
Thrombin	(LVPR↓GS)
PreScission	(LEVLFQ↓GP).

Also embraced by the term protease cleavage site is an intein, which is a self-cleaving sequence. The self-splicing reaction is controllable, for example by varying the concentration of reducing agent present.

In use, the protease cleavage site is cleaved and the N-terminal region (preferably the N-terminus) of the TM becomes exposed. The resulting polypeptide has a TM with an N-terminal domain or an intra domain that is substantially free from the remainder of the fusion protein. This arrangement ensures that the N-terminal component (or intra domain) of the TM may interact directly with a Binding Site on a target cell.

In a preferred embodiment, the TM and the protease cleavage site are distanced apart in the fusion protein by at most 10 amino acid residues, more preferably by at most 5 amino acid residues, and most preferably by zero amino acid residues. Thus, following cleavage of the protease cleavage site, a fusion is provided with a TM that has an N-terminal domain that is substantially free from the remainder of the fusion. This arrangement ensures that the N-terminal component of the Targeting Moiety may interact directly with a Binding Site on a target cell.

One advantage associated with the above-mentioned activation step is that the TM only becomes susceptible to N-terminal degradation once proteolytic cleavage of the fusion protein has occurred. In addition, the selection of a specific protease cleavage site permits selective activation of the polypeptide fusion into a di-chain conformation.

Construction of the single-chain polypeptide fusion of the present invention places the protease cleavage site between the TM and the non-cytotoxic protease component.

It is preferred that, in the single-chain fusion, the TM is located between the protease cleavage site and the translocation component. This ensures that the TM is attached to the translocation domain (i.e. as occurs with native clostridial holotoxin), though in the case of the present invention the order of the two components is reversed *vis-à-vis* native holotoxin. A further advantage with this arrangement is that the TM is located in an exposed loop region of the fusion protein, which has minimal structural effects on the conformation of the fusion protein. In this regard, said loop is variously referred to as the linker, the activation loop, the inter-domain linker, or just the surface exposed loop (Schiavo *et al* 2000, Phys. Rev., 80, 717-766; Turton *et al.*, 2002, Trends Biochem. Sci., 27, 552-558).

In one embodiment, in the single chain polypeptide, the non-cytotoxic protease component and the translocation component are linked together by a disulphide

bond. Thus, following cleavage of the protease cleavage site, the polypeptide assumes a di-chain conformation, wherein the protease and translocation components remain linked together by the disulphide bond. To this end, it is preferred that the protease and translocation components are distanced apart from one another in the single chain fusion protein by a maximum of 100 amino acid residues, more preferably a maximum of 80 amino acid residues, particularly preferably by a maximum of 60 amino acid residues, and most preferably by a maximum of 50 amino acid residues.

In one embodiment, the non-cytotoxic protease component forms a disulphide bond with the translocation component of the fusion protein. For example, the amino acid residue of the protease component that forms the disulphide bond is located within the last 20, preferably within the last 10 C-terminal amino acid residues of the protease component. Similarly, the amino acid residue within the translocation component that forms the second part of the disulphide bond may be located within the first 20, preferably within the first 10 N-terminal amino acid residues of the translocation component.

Alternatively, in the single chain polypeptide, the non-cytotoxic protease component and the TM may be linked together by a disulphide bond. In this regard, the amino acid residue of the TM that forms the disulphide bond is preferably located away from the N-terminus of the TM, more preferably towards to C-terminus of the TM.

In one embodiment, the non-cytotoxic protease component forms a disulphide bond with the TM component of the fusion protein. In this regard, the amino acid residue of the protease component that forms the disulphide bond is preferably located within the last 20, more preferably within the last 10 C-terminal amino acid residues of the protease component. Similarly, the amino acid residue within the TM component that forms the second part of the disulphide bond is preferably located within the last 20, more preferably within the last 10 C-terminal amino acid residues of the TM.

The above disulphide bond arrangements have the advantage that the protease and translocation components are arranged in a manner similar to that for native clostridial neurotoxin. By way of comparison, referring to the primary amino acid sequence for native clostridial neurotoxin, the respective cysteine amino acid residues are distanced apart by between 8 and 27 amino acid residues – taken from Popoff, MR & Marvaud, J-C, 1999, Structural & genomic features of clostridial neurotoxins, Chapter 9, in The Comprehensive Sourcebook of Bacterial Protein Toxins. Ed. Alouf & Freer:

Serotype[1]	Sequence	'Native' length between C-C
BoNT/A1	CVRGIITSKTKS---LDKGYNKALNDLC	23
BoNT/A2	CVRGIIPFKTKS---LDEGYNKALNDLC	23
BoNT/B	CKSVKAPG----------IC	8
BoNT/C	CHKAIDGRS--------LYNKTLDC	15
BoNT/D	CLRLTK----------NSRDDSTC	12
BoNT/E	CKN-IVSVK--------GIRK---SIC	13
BoNT/F	CKS-VIPRK--------GTKAPP-RLC	15
BoNT/G	CKPVMYKNT--------GKSE---QC	13
TeNT	CKKIIPPTNIRENLYNRTASLTDLGGELC	27

[1]Information from proteolytic strains only

The fusion protein may comprise one or more purification tags, which are located N-terminal to the protease component and/ or C-terminal to the translocation component.

Whilst any purification tag may be employed, the following are preferred:

His-tag (e.g. 6 × histidine), preferably as a C-terminal and/ or N-terminal tag

MBP-tag (maltose binding protein), preferably as an N-terminal tag
GST-tag (glutathione-S-transferase), preferably as an N-terminal tag
His-MBP-tag, preferably as an N-terminal tag
GST-MBP-tag, preferably as an N-terminal tag
Thioredoxin-tag, preferably as an N-terminal tag
CBD-tag (Chitin Binding Domain), preferably as an N-terminal tag.

According to a further embodiment of the present invention, one or more peptide spacer molecules may be included in the fusion protein. For example, a peptide spacer may be employed between a purification tag and the rest of the fusion protein molecule (e.g. between an N-terminal purification tag and a protease component of the present invention; and/ or between a C-terminal purification tag and a translocation component of the present invention). A peptide spacer may be also employed between the TM and translocation components of the present invention.

A variety of different spacer molecules may be employed in any of the fusion proteins of the present invention. Examples of such spacer molecules include those illustrated in Figures 28 and 29. Particular mention here is made to GS15, GS20, GS25, and Hx27 – see Figures 28 and 29.

The present inventors have unexpectedly found that the fusion proteins (eg. CPNv/A) of the present invention may demonstrate an improved binding activity for nociceptive sensory afferents when the size of the spacer is selected so that (in use) the C-terminus of the TM and the N-terminus of the translocation component are separated from one another by 40-105 angstroms, preferably by 50-100 angstroms, and more preferably by 50-90 angstroms. In another embodiment, the preferred spacers have an amino acid sequence of 11-29 amino acid residues, preferably 15-27 amino acid residues, and more preferably 20-27 amino acid residues. Suitable spacers may be routinely identified and obtained according to Crasto, C.J. and Feng, J.A. (2000) May, 13(5), pp. 309-312 – see also http://www.fccc.edu/research/labs/feng/limker.html.

In accordance with a second aspect of the present invention, there is provided a DNA sequence that encodes the above-mentioned single chain polypeptide. In a preferred aspect of the present invention, the DNA sequence is prepared as part of a DNA vector, wherein the vector comprises a promoter and terminator.

In a preferred embodiment, the vector has a promoter selected from:

Promoter	Induction Agent	Typical Induction Condition
Tac (hybrid)	IPTG	0.2 mM (0.05-2.0mM)
AraBAD	L-arabinose	0.2% (0.002-0.4%)
T7-*lac* operator	IPTG	0.2 mM (0.05-2.0mM)

The DNA construct of the present invention is preferably designed *in silico*, and then synthesised by conventional DNA synthesis techniques.

The above-mentioned DNA sequence information is optionally modified for codon-biasing according to the ultimate host cell (e.g. *E. coli*) expression system that is to be employed.

The DNA backbone is preferably screened for any inherent nucleic acid sequence, which when transcribed and translated would produce an amino acid sequence corresponding to the protease cleave site encoded by the second peptide-coding sequence. This screening may be performed manually or with the assistance of computer software (e.g. the MapDraw program by DNASTAR, Inc.).

According to a further embodiment of the present invention, there is provided a method of preparing a non-cytotoxic agent, comprising:

a. contacting a single-chain polypeptide fusion protein of the invention with a protease capable of cleaving the protease cleavage site;

b. cleaving the protease cleavage site, and thereby forming a di-chain fusion protein.

This aspect provides a di-chain polypeptide, which generally mimics the structure of clostridial holotoxin. In more detail, the resulting di-chain polypeptide typically has a structure wherein:

a. the first chain comprises the non-cytotoxic protease, or a fragment thereof, which protease or protease fragment is capable of cleaving a protein of the exocytic fusion apparatus of a nociceptive sensory afferent;

b. the second chain comprises the TM and the translocation domain that is capable of translocating the protease or protease fragment from within an endosome, across the endosomal membrane and into the cytosol of the nociceptive sensory afferent; and

the first and second chains are disulphide linked together.

In use, the single chain or di-chain polypeptide of the invention treat, prevent or ameliorate pain.

In use, a therapeutically effective amount of a single chain or di-chain polypeptide of the invention is administered to a patient.

The present invention addresses a wide range of pain conditions, in particular chronic pain conditions. Preferred conditions include cancerous and non-cancerous pain, inflammatory pain and neuropathic pain. The opioid-fusions of the present application are particularly suited to addressing inflammatory pain, though may be less suited to addressing neuropathic pain. The galanin-fusions are more suited to addressing neuropathic pain.

In use, the polypeptides of the present invention are typically employed in the form of a pharmaceutical composition in association with a pharmaceutical

carrier, diluent and/or excipient, although the exact form of the composition may be tailored to the mode of administration. Administration is preferably to a mammal, more preferably to a human.

The polypeptides may, for example, be employed in the form of a sterile solution for intra-articular administration or intra-cranial administration. Spinal injection (e.g. epidural or intrathecal) is preferred.

The dosage ranges for administration of the polypeptides of the present invention are those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the components, the route of administration, the nature of the formulation, the age of the patient, the nature, extent or severity of the patient's condition, contraindications, if any, and the judgement of the attending physician.

Suitable daily dosages are in the range 0.0001-1 mg/kg, preferably 0.0001-0.5 mg/kg, more preferably 0.002-0.5 mg/kg, and particularly preferably 0.004-0.5 mg/kg. The unit dosage can vary from less that 1 microgram to 30mg, but typically will be in the region of 0.01 to 1 mg per dose, which may be administered daily or preferably less frequently, such as weekly or six monthly.

A particularly preferred dosing regimen is based on 2.5 ng of fusion protein (e.g. CPNv/A) as the 1X dose. In this regard, preferred dosages are in the range 1X–100X (i.e. 2.5-250 ng). This dosage range is significantly lower (i.e. at least 10-fold, typically 100-fold lower) than would be employed with other types of analgesic molecules such as NSAIDS, morphine, and gabapentin. Moreover, the above-mentioned difference is considerably magnified when the same comparison is made on a molar basis – this is because the fusion proteins of the present invention have a considerably greater Mw than do conventional 'small' molecule therapeutics.

Wide variations in the required dosage, however, are to be expected depending on the precise nature of the components, and the differing efficiencies of various routes of administration.

Variations in these dosage levels can be adjusted using standard empirical routines for optimisation, as is well understood in the art.

Compositions suitable for injection may be in the form of solutions, suspensions or emulsions, or dry powders which are dissolved or suspended in a suitable vehicle prior to use.

Fluid unit dosage forms are typically prepared utilising a pyrogen-free sterile vehicle. The active ingredients, depending on the vehicle and concentration used, can be either dissolved or suspended in the vehicle.

In preparing administrable solutions, the polypeptides can be dissolved in a vehicle, the solution being made isotonic if necessary by addition of sodium chloride and sterilised by filtration through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Alternatively, if solution stability is adequate, the solution in its sealed containers may be sterilised by autoclaving.

Advantageously additives such as buffering, solubilising, stabilising, preservative or bactericidal, suspending or emulsifying agents may be dissolved in the vehicle.

Dry powders which are dissolved or suspended in a suitable vehicle prior to use may be prepared by filling pre-sterilised drug substance and other ingredients into a sterile container using aseptic technique in a sterile area.

Alternatively the polypeptides and other ingredients may be dissolved in an aqueous vehicle, the solution is sterilized by filtration and distributed into suitable

containers using aseptic technique in a sterile area. The product is then freeze dried and the containers are sealed aseptically.

Parenteral suspensions, suitable for intramuscular, subcutaneous or intradermal injection, are prepared in substantially the same manner, except that the sterile components are suspended in the sterile vehicle, instead of being dissolved and sterilisation cannot be accomplished by filtration. The components may be isolated in a sterile state or alternatively it may be sterilised after isolation, e.g. by gamma irradiation.

Advantageously, a suspending agent for example polyvinylpyrrolidone is included in the composition/s to facilitate uniform distribution of the components.

Definitions Section

Targeting Moiety (TM) means any chemical structure associated with an agent that functionally interacts with a Binding Site to cause a physical association between the agent and the surface of a target cell. In the context of the present invention, the target cell is a nociceptive sensory afferent. The term TM embraces any molecule (i.e. a naturally occurring molecule, or a chemically/physically modified variant thereof) that is capable of binding to a Binding Site on the target cell, which Binding Site is capable of internalisation (e.g. endosome formation) - also referred to as receptor-mediated endocytosis. The TM may possess an endosomal membrane translocation function, in which case separate TM and Translocation Domain components need not be present in an agent of the present invention.

The TM of the present invention binds (preferably specifically binds) to a nociceptive sensory afferent (e.g. a primary nociceptive afferent). In this regard, specifically binds means that the TM binds to a nociceptive sensory afferent (e.g. a primary nociceptive afferent) with a greater affinity than it binds to other neurons such as non-nociceptive afferents, and/ or to motor neurons (i.e. the

natural target for clostridial neurotoxin holotoxin). The term "specifically binding" can also mean that a given TM binds to a given receptor, for example the ORL_1 receptor, with a binding affinity (Ka) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably, 10^9 M^{-1} or greater.

For the purposes of this invention, an agonist is defined as a molecule that is capable of stimulating the process of exocytic fusion in a target cell, which process is susceptible to inhibition by a protease (or fragment thereof) capable of cleaving a protein of the exocytic fusion apparatus in said target cell.

Accordingly, the particular agonist definition of the present invention would exclude many molecules that would be conventionally considered as agonists.

For example, nerve growth factor (NGF) is an agonist in respect of its ability to promote neuronal differentiation via binding to a TrkA receptor. However, NGF is not an agonist when assessed by the above criteria because it is not a principal inducer of exocytic fusion. In addition, the process that NGF stimulates (i.e. cell differentiation) is not susceptible to inhibition by the protease activity of a non-cytotoxic toxin molecule.

The term "fragment", when used in relation to a protein, means a peptide having at least thirty-five, preferably at least twenty-five, more preferably at least twenty, and most preferably at least ten amino acid residues of the protein in question.

The term "variant", when used in relation to a protein, means a peptide or peptide fragment of the protein that contains one or more analogues of an amino acid (e.g. an unnatural amino acid), or a substituted linkage.

The term "derivative", when used in relation to a protein, means a protein that comprises the protein in question, and a further peptide sequence. The further peptide sequence should preferably not interfere with the basic folding and thus

conformational structure of the original protein. Two or more peptides (or fragments, or variants) may be joined together to form a derivative. Alternatively, a peptide (or fragment, or variant) may be joined to an unrelated molecule (e.g. a second, unrelated peptide). Derivatives may be chemically synthesized, but will be typically prepared by recombinant nucleic acid methods. Additional components such as lipid, and/or polysaccharide, and/or polyketide components may be included.

Throughout this specification, reference to the "ORL_1 receptor" embraces all members of the ORL_1 receptor family. Members of the ORL_1 receptor family typically have a seven transmembrane domain structure and are coupled to G-proteins of the G_i and G_0 families. A method for determining the G-protein-stimulating activity of ligands of the ORL_1 receptor is given in Example 12. A method for measuring reduction in cellular cAMP levels following ORL_1 activation is given in Example 11. A further characteristic of members of the ORL_1 receptor family is that they are typically able to bind nociceptin (the natural ligand of ORL_1). As an example, all alternative splice variants of the ORL_1 receptor, are members of the ORL_1 receptor family.

The term non-cytotoxic means that the protease molecule in question does not kill the target cell to which it has been re-targeted.

The protease of the present invention embraces all naturally-occurring non-cytotoxic proteases that are capable of cleaving one or more proteins of the exocytic fusion apparatus in eukaryotic cells.

The protease of the present invention is preferably a bacterial protease (or fragment thereof). More preferably the bacterial protease is selected from the genera *Clostridium* or *Neisseria* (e.g. a clostridial L-chain, or a neisserial IgA protease preferably from *N. gonorrhoeae*).

The present invention also embraces modified non-cytotoxic proteases, which include amino acid sequences that do not occur in nature and/or synthetic amino acid residues, so long as the modified proteases still demonstrate the above-mentioned protease activity.

The protease of the present invention preferably demonstrates a serine or metalloprotease activity (e.g. endopeptidase activity). The protease is preferably specific for a SNARE protein (e.g. SNAP-25, synaptobrevin/VAMP, or syntaxin).

Particular mention is made to the protease domains of neurotoxins, for example the protease domains of bacterial neurotoxins. Thus, the present invention embraces the use of neurotoxin domains, which occur in nature, as well as recombinantly prepared versions of said naturally-occurring neurotoxins.

Exemplary neurotoxins are produced by clostridia, and the term clostridial neurotoxin embraces neurotoxins produced by *C. tetani* (TeNT), and by *C. botulinum* (BoNT) serotypes A-G, as well as the closely related BoNT-like neurotoxins produced by *C. baratii* and *C. butyricum*. The above-mentioned abbreviations are used throughout the present specification. For example, the nomenclature BoNT/A denotes the source of neurotoxin as BoNT (serotype A). Corresponding nomenclature applies to other BoNT serotypes.

The term L-chain fragment means a component of the L-chain of a neurotoxin, which fragment demonstrates a metalloprotease activity and is capable of proteolytically cleaving a vesicle and/or plasma membrane associated protein involved in cellular exocytosis.

A Translocation Domain is a molecule that enables translocation of a protease (or fragment thereof) into a target cell such that a functional expression of protease activity occurs within the cytosol of the target cell. Whether any molecule (e.g. a protein or peptide) possesses the requisite translocation function of the present invention may be confirmed by any one of a number of conventional assays.

For example, Shone C. (1987) describes an *in vitro* assay employing liposomes, which are challenged with a test molecule. Presence of the requisite translocation function is confirmed by release from the liposomes of K^+ and/or labelled NAD, which may be readily monitored [see Shone C. (1987) Eur. J. Biochem; vol. 167(1): pp. 175-180].

A further example is provided by Blaustein R. (1987), which describes a simple *in vitro* assay employing planar phospholipid bilayer membranes. The membranes are challenged with a test molecule and the requisite translocation function is confirmed by an increase in conductance across said membranes [see Blaustein (1987) FEBS Letts; vol. 226, no. 1: pp. 115-120].

Additional methodology to enable assessment of membrane fusion and thus identification of Translocation Domains suitable for use in the present invention are provided by Methods in Enzymology Vol 220 and 221, Membrane Fusion Techniques, Parts A and B, Academic Press 1993.

The Translocation Domain is preferably capable of formation of ion-permeable pores in lipid membranes under conditions of low pH. Preferably it has been found to use only those portions of the protein molecule capable of pore-formation within the endosomal membrane.

The Translocation Domain may be obtained from a microbial protein source, in particular from a bacterial or viral protein source. Hence, in one embodiment, the Translocation Domain is a translocating domain of an enzyme, such as a bacterial toxin or viral protein.

It is well documented that certain domains of bacterial toxin molecules are capable of forming such pores. It is also known that certain translocation domains of virally expressed membrane fusion proteins are capable of forming such pores. Such domains may be employed in the present invention.

The Translocation Domain may be of a clostridial origin, namely the H_N domain (or a functional component thereof). H_N means a portion or fragment of the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It is preferred that the H-chain substantially lacks the natural binding function of the H_C component of the H-chain. In this regard, the H_C function may be removed by deletion of the H_C amino acid sequence (either at the DNA synthesis level, or at the post-synthesis level by nuclease or protease treatment). Alternatively, the H_C function may be inactivated by chemical or biological treatment. Thus, the H-chain is preferably incapable of binding to the Binding Site on a target cell to which native clostridial neurotoxin (i.e. holotoxin) binds.

In one embodiment, the translocation domain is a H_N domain (or a fragment thereof) of a clostridial neurotoxin. Examples of suitable clostridial Translocation Domains include:

Botulinum type A neurotoxin	- amino acid residues (449-871)
Botulinum type B neurotoxin	- amino acid residues (441-858)
Botulinum type C neurotoxin	- amino acid residues (442-866)
Botulinum type D neurotoxin	- amino acid residues (446-862)
Botulinum type E neurotoxin	- amino acid residues (423-845)
Botulinum type F neurotoxin	- amino acid residues (440-864)
Botulinum type G neurotoxin	- amino acid residues (442-863)
Tetanus neurotoxin	- amino acid residues (458-879)

For further details on the genetic basis of toxin production in *Clostridium botulinum* and *C. tetani*, we refer to Henderson *et al* (1997) in *The Clostridia: Molecular Biology and Pathogenesis, Academic press.*

The term H_N embraces naturally-occurring neurotoxin H_N portions, and modified H_N portions having amino acid sequences that do not occur in nature and/or

synthetic amino acid residues, so long as the modified H_N portions still demonstrate the above-mentioned translocation function.

Alternatively, the Translocation Domain may be of a non-clostridial origin (see Table 4). Examples of non-clostridial Translocation Domain origins include, but not be restricted to, the translocation domain of diphtheria toxin [O=Keefe *et al.,* Proc. Natl. Acad. Sci. USA (1992) 89, 6202-6206; Silverman *et al.,* J. Biol. Chem. (1993) 269, 22524-22532; and London, E. (1992) *Biochem. Biophys. Acta., 1112, pp.25-51*], the translocation domain of *Pseudomonas* exotoxin type A [Prior *et al.* Biochemistry (1992) 31, 3555-3559], the translocation domains of anthrax toxin [Blanke *et al.* Proc. Natl. Acad. Sci. USA (1996) 93, 8437-8442], a variety of fusogenic or hydrophobic peptides of translocating function [Plank *et al.* J. Biol. Chem. (1994) 269, 12918-12924; and Wagner *et al* (1992) *PNAS, 89, pp.7934-7938*], and amphiphilic peptides [Murata *et al* (1992) *Biochem., 31, pp.1986-1992*]. The Translocation Domain may mirror the Translocation Domain present in a naturally-occurring protein, or may include amino acid variations so long as the variations do not destroy the translocating ability of the Translocation Domain.

Particular examples of viral Translocation Domains suitable for use in the present invention include certain translocating domains of virally expressed membrane fusion proteins. For example, Wagner *et al.* (1992) and Murata *et al.* (1992) describe the translocation (i.e. membrane fusion and vesiculation) function of a number of fusogenic and amphiphilic peptides derived from the N-terminal region of influenza virus haemagglutinin. Other virally expressed membrane fusion proteins known to have the desired translocating activity are a translocating domain of a fusogenic peptide of Semliki Forest Virus (SFV), a translocating domain of vesicular stomatitis virus (VSV) glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein. Virally encoded Aspike proteins have particular application in the context of the present invention, for example, the E1 protein of SFV and the G protein of the G protein of VSV.

Use of the Translocation Domains listed in Table (below) includes use of sequence variants thereof. A variant may comprise one or more conservative nucleic acid substitutions and/ or nucleic acid deletions or insertions, with the proviso that the variant possesses the requisite translocating function. A variant may also comprise one or more amino acid substitutions and/ or amino acid deletions or insertions, so long as the variant possesses the requisite translocating function.

Translocation domain source	**Amino acid residues**	**References**
Diphtheria toxin	194-380	**Silverman *et al.***, 1994, J. Biol. Chem. 269, 22524-22532 **London E.**, 1992, Biochem. Biophys. Acta., 1113, 25-51
Domain II of pseudomonas exotoxin	405-613	**Prior *et al.***, 1992, Biochemistry 31, 3555-3559 **Kihara & Pastan**, 1994, Bioconj Chem. 5, 532-538
Influenza virus haemagglutinin	GLFGAIAGFIENGWE GMIDGWYG, and Variants thereof	**Plank *et al.***, 1994, J. Biol. Chem. 269, 12918-12924 **Wagner *et al.***, 1992, PNAS, 89, 7934-7938 **Murata *et al.***, 1992, Biochemistry 31, 1986-1992
Semliki Forest virus fusogenic protein	Translocation domain	**Kielian *et al.***, 1996, J Cell Biol. 134(4), 863-872
Vesicular Stomatitis virus glycoprotein G	118-139	**Yao *et al.***, 2003, Virology 310(2), 319-332
SER virus F protein	Translocation domain	**Seth *et al.***, 2003, J Virol 77(11) 6520-6527

Translocation domain source	Amino acid residues	References
Foamy virus envelope glycoprotein	Translocation domain	**Picard-Maureau *et al.***, 2003, J Virol. 77(8), 4722-4730

Figures

Figure 1 Purification of a LC/A-nociceptin-H_N/A fusion protein

Figure 2 Purification of a nociceptin-LC/A-H_N/A fusion protein

Figure 3 Purification of a LC/C-nociceptin-H_N/C fusion protein

Figure 4 Purification of a LC/A-met enkephalin-H_N/A fusion protein

Figure 5 Comparison of binding efficacy of a LC/A-nociceptin-H_N/A fusion protein and a nociceptin-LC/A-H_N/A fusion protein

Figure 6 *In vitro* catalytic activity of a LC/A-nociceptin-H_N/A fusion protein

Figure 7 Purification of a LC/A-nociceptin variant-H_N/A fusion protein

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Figure 10 Inhibition of SP release and cleavage of SNAP-25 by CPN-A

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Figure 14 CPNv-A fusion-mediated displacement of [3H]-nociceptin binding

Figure 15 Expressed / purified CPNv(Ek)-A product

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Figure 18 Cleavage of syntaxin by CPNv-C

Figure 19 CPN-A efficacy in the Acute Capsaicin-Induced Mechanical Allodynia model

Figure 20 CPN-A efficacy in the Streptozotocin (STZ)-Induced Peripheral Diabetic Neuropathy (Neuropathic Pain) model

Figure 21 CPNv-A efficacy in the Acute Capsaicin-Induced Mechanical Allodynia model

Figure 22 Expressed / purified LC/A-CPLE-H_N/A product

Figure 23 Expressed / purified LC/A-CPBE-H_N/A product

Figure 24 Expressed / purified CPOP-A product

Figure 25 Expressed / purified CPOPv-A product

Figure 26 *In vitro* SNAP-25 cleavage in a DRG cell model

Figure 27 Expressed / purified CPNv-A-FXa-HT (removable his-tag)

Figure 28 *In vitro* efficacy of LC/A-nociceptin-H_N/A fusion proteins with variable spacer length, as assessed by ligand competition assay

Figure 29 *In vitro* efficacy of LC/A-nociceptin-H_N/A fusion proteins with variable spacer length, as assessed by *in vitro* SNAP-25 cleavage

The Figures are now described in more detail.

Figure 1 - Purification of a LC/A-nociceptin-H_N/A fusion protein

Using the methodology outlined in Example 9, a LC/A-nociceptin-H_N/A fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE (Panel A) and Western blotting (Panel B). Anti-nociceptin antisera (obtained from Abcam) were used as the primary antibody for Western blotting. The final purified material in

the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

Figure 2 - Purification of a nociceptin-LC/A-H_N/A fusion protein

Using the methodology outlined in Example 9, a nociceptin-LC/A-H_N/A fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE (Panel A) and Western blotting (Panel B). Anti-nociceptin antisera (obtained from Abcam) were used as the primary antibody for Western blotting. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

Figure 3 - Purification of a LC/C-nociceptin-H_N/C fusion protein

Using the methodology outlined in Example 9, an LC/C-nociceptin-H_N/C fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE (Panel A) and Western blotting (Panel B). Anti-nociceptin antisera (obtained from Abcam) were used as the primary antibody for Western blotting. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

Figure 4 - Purification of a LC/A-met enkephalin-H_N/A fusion protein

Using the methodology outlined in Example 9, an LC/A-met enkephalin-H_N/A fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

Figure 5 - Comparison of binding efficacy of a LC/A-nociceptin-H_N/A fusion protein and a nociceptin-LC/A-H_N/A fusion protein

The ability of nociceptin fusions to bind to the ORL_1 receptor was assessed using a simple competition-based assay. Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of test material in the presence of 1 nM [3H]-nociceptin. The reduction in specific binding of the radiolabelled ligand was assessed by scintillation counting, and plotted in comparison to the efficacy of unlabelled ligand (Tocris nociceptin). It is clear that the LC/A-nociceptin-H_N/A fusion is far superior to the nociceptin-LC/A-H_N/A fusion at interacting with the ORL_1 receptor.

Figure 6 - *In vitro* catalytic activity of a LC/A-nociceptin-H_N/A fusion protein

The *in vitro* endopeptidase activity of the purified LC/A-nociceptin-H_N/A fusion protein was determined essentially as described in Chaddock *et al* 2002, Prot. Express Purif. 25, 219-228. Briefly, SNAP-25 peptide immobilised to an ELISA plate was exposed to varying concentrations of fusion protein for 1 hour at 37°C. Following a series of washes, the amount of cleaved SNAP-25 peptide was quantified by reactivity with a specific antisera.

Figure 7 - Purification of a LC/A-nociceptin variant-H_N/A fusion protein

Using the methodology outlined in Example 9, an LC/A-nociceptin variant-H_N/A fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

Figure 8 - Comparison of binding efficacy of a LC/A-nociceptin-H_N/A fusion protein and a LC/A-nociceptin variant-H_N/A fusion protein

The ability of nociceptin fusions to bind to the ORL_1 receptor was assessed using a simple competition-based assay. Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of test material in the presence of 1nM [3H]-nociceptin. The reduction in specific binding of the radiolabelled ligand was assessed by scintillation counting, and plotted in comparison to the efficacy of unlabelled ligand (Tocris nociceptin). It is clear that the LC/A-nociceptin variant-H_N/A fusion (CPNv-LHA) is superior to the LC/A-nociceptin variant-H_N/A fusion (CPN-LHA) at interacting with the ORL_1 receptor.

Figure 9 - Expressed / purified LC/A-nociceptin-H_N/A fusion protein family with variable spacer length product(s)

Using the methodology outlined in Example 9, variants of the LC/A-CPN-H_N/A fusion consisting of GS10, GS30 and HX27 are purified from *E. coli* cell paste. Samples from the purification of LC/A-CPN(GS10)-H_N/A, LC/A-CPN(GS15)-H_N/A, LC/A-CPN(GS25)-H_N/A, LC/A-CPN(GS30)-H_N/A and LC/A-CPN(HX27)-H_N/A were assessed by SDS-PAGE prior to staining with Coomassie Blue. The

electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPBE-A. Top panel: M = benchmark molecular mass markers; S = total *E. coli* protein soluble fraction; FT = proteins that did not bind to the Ni^{2+}-charged Sepharose column; FUSION = fusion protein eluted by the addition of imidazole. Bottom panel: Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni^{2+}-charged Sepharose; Lane 4 = Factor Xa treated material prior to final capture on Ni^{2+}-charged Sepharose; Lane 5 = purified final material post activation with Factor Xa (5 µl); Lane 6 = purified final material post activation with Factor Xa (10 µl); Lane 7 = purified final material post activation with Factor Xa (20 µl); Lane 8 = purified final material post activation with Factor Xa + DTT (5 µl); Lane 9 = purified final material post activation with Factor Xa + DTT (10 µl); Lane 10 = purified final material post activation with Factor Xa + DTT (20 µl).

Figure 10 - Inhibition of SP release and cleavage of SNAP-25 by CPN-A

Briefly, primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPN-A for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis and plotted against fusion concentration (dashed line). Material was also recovered for an analysis of substance P content using a specific EIA kit. Inhibition of substance P release is illustrated by the solid line. The fusion concentration required to achieve 50% maximal SNAP-25 cleavage is estimated to be 6.30±2.48 nM.

Figure 11 - Inhibition of SP release and cleavage of SNAP-25 over extended time periods after exposure of DRG to CPN-A

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPN-A for 24 hours. Botulinum neurotoxin (BoNT/A) was used

as a control. After this initial exposure, extracellular material was removed by washing, and the cells incubated at 37°C for varying periods of time. At specific time points, cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis and illustrated by the dotted lines. Material was also recovered for an analysis of substance P content using a specific EIA kit. Inhibition of substance P release is illustrated by the solid lines.

Figure 12 - Cleavage of SNAP-25 by CPNv-A

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPNv-A for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis. The fusion concentration required to achieve 50% maximal SNAP-25 cleavage is estimated to be 1.38±0.36 nM.

Figure 13 - Cleavage of SNAP-25 over extended time periods after exposure of DRG to CPNv-A

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPNv-A for 24 hours. CPN-A was used as a control. After this initial exposure, extracellular material was removed by washing, and the cells incubated at 37°C for varying periods of time. At specific time points, cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis.

Figure 14 - CPNv-A fusion-mediated displacement of [3H]-nociceptin binding

The ability of nociceptin fusions to bind to the ORL_1 receptor was assessed using a simple competition-based assay. Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of test material in the presence of 1 nM [3H]-nociceptin. The reduction in specific binding of the radiolabelled ligand was assessed by scintillation counting, and plotted in comparison to the efficacy of unlabelled ligand (Tocris nociceptin). It is clear that the LC/A-nociceptin variant-H_N/A fusion (labelled as CPNv-LHnA) is superior to the LC/A-nociceptin-H_N/A fusion (labelled as CPN-LHnA) at interacting with the ORL_1 receptor.

Figure 15 - Expressed / purified CPNv(Ek)-A product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPNv(Ek)-A. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni^{2+}-charged Sepharose; Lane 4 = purified final material post activation with enterokinase (5 µl); Lane 5 = purified final material post activation with enterokinase (10 µl); Lane 6 = purified final material post activation with enterokinase (20 µl); Lane 7 = purified final material post activation with enterokinase + DTT (5 µl); Lane 8 = purified final material post activation with enterokinase + DTT (10 µl); Lane 9 = purified final material post activation with enterokinase + DTT (20 µl).

Figure 16 - Cleavage of SNAP-25 by CPNv(Ek)-A

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPNv(Ek)-A for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis. CPNv-A as prepared in Example 9 was used for comparison purposes. The percentage cleavage of SNAP-25 by

CPNv(Ek)-A (labelled as En activated) and CPNv-A (labelled as Xa activated) are illustrated.

Figure 17 - Expressed / purified CPNv-C product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPNv-C. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni^{2+}-charged Sepharose; Lane 4 = Factor Xa treated material prior to final capture on Ni^{2+}-charged Sepharose; Lane 5 = purified material following second capture on Ni^{2+}-charged Sepharose; Lane 6 = final purified material; Lane 7 = final purified material + DTT; Lane 8 = benchmark molecular mass markers.

Figure 18 - Cleavage of syntaxin by CPNv-C

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPNv-C for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-syntaxin to facilitate an assessment of syntaxin cleavage. The percentage of cleaved syntaxin was calculated by densitometric analysis. The fusion concentration required to achieve 50% maximal syntaxin cleavage is estimated to be 3.13±1.96 nM.

Figure 19 - CPN-A efficacy in the Acute Capsaicin-Induced Mechanical Allodynia model

The ability of an LC/A-nociceptin-H_N/A fusion (CPN/A) to inhibit capsaicin-induced mechanical allodynia was evaluated following subcutaneous intraplantar injection in the rat hind paw. Test animals were evaluated for paw withdrawal frequency (PWF%) in response to a 10 g Von Frey filament stimulus series (10 stimuli x 3 trials) prior to recruitment into the study (Pre-Treat); after subcutaneous

intraplantar treatment with CPN/A but before capsaicin (Pre-CAP); and following capsaicin challenge post-injection of CPN/A (average of responses at 15' and 30'; CAP). Capsaicin challenge was achieved by injection of 10 µL of a 0.3% solution. Sample dilutions were prepared in 0.5% BSA/saline.

Figure 20 - CPN-A efficacy in the Streptozotocin (STZ)-Induced Peripheral Diabetic Neuropathy (Neuropathic Pain) model

Male Sprague-Dawley rats (250-300 g) are treated with 65 mg/kg STZ in citrate buffer (I.V.) and blood glucose and lipid are measured weekly to define the readiness of the model. Paw Withdrawal Threshold (PWT) is measured in response to a Von Frey filament stimulus series over a period of time. Allodynia is said to be established when the PWT on two consecutive test dates (separated by 1 week) measures below 6 g on the scale. At this point, rats are randomized to either a saline group (negative efficacy control), gabapentin group (positive efficacy control) or a test group (CPN/A). Test materials (20-25 µl) are injected subcutaneously as a single injection (except gabapentin) and the PWT is measured at 1 day post-treatment and periodically thereafter over a 2 week period. Gabapentin (30 mg/kg i.p. @ 3 ml/kg injection volume) is injected daily, 2 hours prior to the start of PWT testing.

Figure 21 - CPNv-A efficacy in the Acute Capsaicin-Induced Mechanical Allodynia model

The ability of an LC/A-nociceptin variant-H_N/A fusion (CPNv/A) to inhibit capsaicin-induced mechanical allodynia was evaluated following subcutaneous intraplantar injection in the rat hind paw. Test animals were evaluated for paw withdrawal frequency (PWF%) in response to a 10 g Von Frey filament stimulus

series (10 stimuli x 3 trials) prior to recruitment into the study (Pre-Treat), after subcutaneous intraplantar treatment with CPNv/A but before capsaicin (Pre-CAP), and following capsaicin challenge post-injection of CPNv/A (average of responses at 15' and 30'; CAP). Capsaicin challenge was achieved by injection of 10 µL of a 0.3% solution. Sample dilutions were prepared in 0.5% BSA/saline. These data are expressed as a normalized paw withdrawal frequency differential, in which the difference between the peak response (post-capsaicin) and the baseline response (pre-capsaicin) is expressed as a percentage. With this analysis, it can be seen that CPNv/A is more potent than CPN/A since a lower dose of CPNv/A is required to achieve similar analgesic effect to that seen with CPN/A.

Figure 22 - Expressed / purified LC/A-CPLE-H_N/A product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPLE-A. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni^{2+}-charged Sepharose; Lane 4 = Factor Xa treated material prior to final capture on Ni^{2+}-charged Sepharose; Lane 5 = purified material following second capture on Ni^{2+}-charged Sepharose; Lane 6 = final purified material; Lane 7 = final purified material + DTT.

Figure 23 - Expressed / purified LC/A-CPBE-H_N/A product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPBE-A. Lane 1 = total *E. coli* protein soluble fraction; Lane 2 = purified material following initial capture on Ni^{2+}-charged Sepharose; Lane 3 = Factor Xa treated material prior to final capture on Ni^{2+}-charged Sepharose; Lane 4 = purified final material post activation with Factor Xa (5 µl); Lane 5 = purified final material post activation with Factor Xa (10

µl); Lane 6 = purified final material post activation with Factor Xa (20 µl); Lane 7 = purified final material post activation with Factor Xa + DTT (5 µl); Lane 8 = purified final material post activation with Factor Xa + DTT (10 µl); Lane 9 = purified final material post activation with Factor Xa + DTT (20 µl); Lane 10 = benchmark molecular mass markers.

Figure 24 - Expressed / purified CPOP-A product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPOP-A. Lane 1 = benchmark molecular mass markers; Lane 2 = purified material following initial capture on Ni^{2+}-charged Sepharose; Lane 3 = Factor Xa treated material prior to final capture on Ni^{2+}-charged Sepharose; Lane 4 = purified material following second capture on Ni^{2+}-charged Sepharose; Lane 5 = purified final material post activation with Factor Xa (5 µl); Lane 6 = purified final material post activation with Factor Xa (10 µl); Lane 7 = purified final material post activation with Factor Xa (20 µl); Lane 8 = purified final material post activation with Factor Xa + DTT (5 µl); Lane 9 = purified final material post activation with Factor Xa + DTT (10 µl); Lane 10 = purified final material post activation with Factor Xa + DTT (20 µl).

Figure 25 - Expressed / purified CPOPv-A product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPOPv-A. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni^{2+}-charged Sepharose; Lane 4 = Factor Xa treated material prior to final capture on Ni^{2+}-charged Sepharose;

Lane 5 = purified final material post activation with Factor Xa (5 µl); Lane 6 = purified final material post activation with Factor Xa (10 µl); Lane 7 = purified final material post activation with Factor Xa (20 µl); Lane 8 = purified final material post activation with Factor Xa + DTT (5 µl); Lane 9 = purified final material post activation with Factor Xa + DTT (10 µl); Lane 10 = purified final material post activation with Factor Xa + DTT (20 µl).

Figure 26 - *In vitro* SNAP-25 cleavage in a DRG cell model

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPOPv-A for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis.

Figure 27 - Expressed / purified CPNv-A-FXa-HT (removable his-tag)

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPNv-A-FXa-HT. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = Factor Xa treated material prior to final capture on Ni^{2+}-charged Sepharose; Lane 4 = purified final material post activation with Factor Xa; Lane 5 = purified final material post activation with Factor Xa + DTT.

Figure 28 - *In vitro* efficacy of LC/A-nociceptin-H_N/A fusion proteins with variable spacer length, as assessed by ligand competition assay

The ability of LC/A-nociceptin-H_N/A fusions of variable spacer length to bind to the ORL_1 receptor was assessed using a simple competition-based assay.

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of test material in the presence of 1 nM [3H]-nociceptin. The reduction in specific binding of the radiolabelled ligand was assessed by scintillation counting, and plotted in comparison to the efficacy of unlabelled ligand (Tocris nociceptin). The upper panel illustrates the displacement characteristics of the GS0, GS20, GS30 and Hx27 spacers, whilst the lower panel illustrates the displacement achieved by the GS10, GS15 and GS25 spaced fusion proteins. It is concluded that the GS0 and GS30 spacers are ineffective, and the GS10 is poorly effective, at displacing nociceptin from the ORL1 receptor.

Figure 29 - *In vitro* efficacy of LC/A-nociceptin-H_N/A fusion proteins with variable spacer length, as assessed by *in vitro* SNAP-25 cleavage

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPN-A (of variable spacer length) for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis. The poorly effective binding characteristics of the GS10 spaced fusion protein (see Figure 28) are reflected in the higher concentrations of fusion required to achieve cleavage of intracellular SNAP-25. GS0 and GS30 spaced fusion proteins were completely ineffective (date not shown). GS15, 20 and 25 spaced fusion proteins were similarly effective.

SEQ ID NOs

SEQ ID1 DNA sequence of the LC/A
SEQ ID2 DNA sequence of the H_N/A
SEQ ID3 DNA sequence of the LC/B
SEQ ID4 DNA sequence of the H_N/B
SEQ ID5 DNA sequence of the LC/C

SEQ ID6 DNA sequence of the H_N/C

SEQ ID7 DNA sequence of the CPN-A linker

SEQ ID8 DNA sequence of the A linker

SEQ ID9 DNA sequence of the N-terminal presentation nociceptin insert

SEQ ID10 DNA sequence of the CPN-C linker

SEQ ID11 DNA sequence of the CPBE-A linker

SEQ ID12 DNA sequence of the CPNvar-A linker

SEQ ID13 DNA sequence of the LC/A-CPN-H_N/A fusion

SEQ ID14 Protein sequence of the LC/A-CPN-H_N/A fusion

SEQ ID15 DNA sequence of the N-LC/A-H_N/A fusion

SEQ ID16 Protein sequence of the N-LC/A-H_N/A fusion

SEQ ID17 DNA sequence of the LC/C-CPN-H_N/C fusion

SEQ ID18 Protein sequence of the LC/C-CPN-H_N/C fusion

SEQ ID19 DNA sequence of the LC/C-CPN-H_N/C (A-linker) fusion

SEQ ID20 Protein sequence of the LC/C-CPN-H_N/C (A-linker) fusion

SEQ ID21 DNA sequence of the LC/A-CPME-H_N/A fusion

SEQ ID22 Protein sequence of the LC/A-CPME-H_N/A fusion

SEQ ID23 DNA sequence of the LC/A-CPBE-H_N/A fusion

SEQ ID24 Protein sequence of the LC/A-CPBE-H_N/A fusion

SEQ ID25 DNA sequence of the LC/A-CPNv-H_N/A fusion

SEQ ID26 Protein sequence of the LC/A-CPNv-H_N/A fusion

SEQ ID27 DNA sequence of the LC/A-CPN[1-11]-HN/A fusion

SEQ ID28 Protein sequence of the LC/A-CPN[1-11]-HN/A fusion

SEQ ID29 DNA sequence of the LC/A-CPN[[Y10]1-11]-HN/A fusion

SEQ ID30 Protein sequence of the LC/A-CPN[[Y10]1-11]-HN/A fusion

SEQ ID31 DNA sequence of the LC/A-CPN[[Y11]1-11]-HN/A fusion

SEQ ID32 Protein sequence of the LC/A-CPN[[Y11]1-11]-HN/A fusion

SEQ ID33 DNA sequence of the LC/A-CPN[[Y14]1-17]-HN/A fusion

SEQ ID34 Protein sequence of the LC/A-CPN[[Y14]1-17]-HN/A fusion

SEQ ID35 DNA sequence of the LC/A-CPN[1-13]-HN/A fusion

SEQ ID36 Protein sequence of the LC/A- CPN[1-13]-HN/A fusion

SEQ ID37 DNA sequence of CPN[1-17]

SEQ ID38 Protein Sequence of CPN[1-17]
SEQ ID39 DNA sequence of CPN[1-11]
SEQ ID40 Protein sequence of CPN[1-11]
SEQ ID41 DNA sequence of CPN[[Y10]1-11]
SEQ ID42 Protein sequence of CPN[[Y10]1-11]
SEQ ID43 DNA sequence of CPN[[Y11]1-11]
SEQ ID44 Protein sequence of CPN[[Y11]1-11]
SEQ ID45 DNA sequence of CPN[[Y14]1-17]
SEQ ID46 Protein sequence of CPN[[Y14]1-17]
SEQ ID47 DNA sequence of CPN[1-13]
SEQ ID48 Protein sequence of CPN[1-13]
SEQ ID49 DNA sequence of CPNv (also known as N[[R14K15]1-17])
SEQ ID50 Protein sequence of CPNv (also known as N[[R14K15]1-17])
SEQ ID51 DNA sequence of the nociceptin-spacer-LC/A-H_N/A fusion
SEQ ID52 Protein sequence of the nociceptin-spacer-LC/A-H_N/A fusion
SEQ ID53 DNA sequence of the CPN-A GS10 linker
SEQ ID54 DNA sequence of the CPN-A GS15 linker
SEQ ID55 DNA sequence of the CPN-A GS25 linker
SEQ ID56 DNA sequence of the CPN-A GS30 linker
SEQ ID57 DNA sequence of the CPN-A HX27 linker
SEQ ID58 DNA sequence of the LC/A-CPN(GS15)-H_N/A fusion
SEQ ID59 Protein sequence of the LC/A-CPN(GS15)-H_N/A fusion
SEQ ID60 DNA sequence of the LC/A-CPN(GS25)-H_N/A fusion
SEQ ID61 Protein sequence of the LC/A-CPN(GS25)-H_N/A fusion
SEQ ID62 DNA sequence of the CPNvar-A Enterokinase activatable linker
SEQ ID63 DNA sequence of the LC/A-CPNv(Ek)-H_N/A fusion
SEQ ID64 Protein sequence of the LC/A-CPNv(Ek)-H_N/A fusion
SEQ ID65 DNA sequence of the CPNvar-A linker
SEQ ID66 DNA sequence of the LC/C-CPNv-H_N/C fusion (act. A)
SEQ ID67 Protein sequence of the LC/C-CPNv-H_N/C fusion (act. A)
SEQ ID68 DNA sequence of the LC/A-CPLE-H_N/A fusion
SEQ ID69 Protein sequence of the LC/A-CPLE-H_N/A fusion

SEQ ID70 DNA sequence of the LC/A-CPOP-H_N/A fusion
SEQ ID71 Protein sequence of the LC/A-CPOP-H_N/A fusion
SEQ ID72 DNA sequence of the LC/A-CPOPv-H_N/A fusion
SEQ ID73 Protein sequence of the LC/A-CPOPv-H_N/A fusion
SEQ ID74 DNA sequence of the IgA protease
SEQ ID75 DNA sequence of the IgA-CPNv-H_N/A fusion
SEQ ID76 Protein sequence of the IgA-CPNv-H_N/A fusion
SEQ ID77 DNA sequence of the FXa-HT
SEQ ID78 DNA sequence of the CPNv-A-FXa-HT
SEQ ID79 Protein sequence of the CPNv-A-FXa-HT fusion
SEQ ID80 DNA sequence of the DT translocation domain
SEQ ID81 DNA sequence of the CPLE-DT-A
SEQ ID82 Protein sequence of the CPLE-DT-A fusion
SEQ ID83 DNA sequence of the TeNT LC
SEQ ID84 DNA sequence of the CPNv-TENT LC
SEQ ID85 Protein sequence of the CPNV-TeNT LC fusion
SEQ ID86 DNA sequence of the CPNvar-C linker
SEQ ID87 DNA sequence of the LC/C-CPNv-H_N/C fusion (act. C)
SEQ ID88 Protein sequence of the LC/C-CPNv-H_N/C fusion (act. C)

Examples

Example 1 - Preparation of a LC/A and H_N/A backbone clones

The following procedure creates the LC and H_N fragments for use as the component backbone for multidomain fusion expression. This example is based on preparation of a serotype A based clone (SEQ ID1 and SEQ ID2), though the procedures and methods are equally applicable to the other serotypes [illustrated by the sequence listing for serotype B (SEQ ID3 and SEQ ID4) and serotype C (SEQ ID5 and SEQ ID6)].

Preparation of cloning and expression vectors

pCR 4 (Invitrogen) is the chosen standard cloning vector, selected due to the lack of restriction sequences within the vector and adjacent sequencing primer sites for easy construct confirmation. The expression vector is based on the pMAL (NEB) expression vector, which has the desired restriction sequences within the multiple cloning site in the correct orientation for construct insertion (*Bam*HI-*Sal*I-*Pst*I-*Hin*dIII). A fragment of the expression vector has been removed to create a non-mobilisable plasmid and a variety of different fusion tags have been inserted to increase purification options.

Preparation of protease (e.g. LC/A) insert

The LC/A (SEQ ID1) is created by one of two ways:

The DNA sequence is designed by back translation of the LC/A amino acid sequence [obtained from freely available database sources such as GenBank (accession number P10845) or Swissprot (accession locus BXA1_CLOBO) using one of a variety of reverse translation software tools (for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)]. *Bam*HI/*Sal*I recognition sequences are incorporated at the 5' and 3' ends respectively of the sequence, maintaining the correct reading frame. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence containing the LC/A open reading frame (ORF) is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

The alternative method is to use PCR amplification from an existing DNA sequence with *Bam*HI and *Sal*I restriction enzyme sequences incorporated into

the 5' and 3' PCR primers respectively. Complementary oligonucleotide primers are chemically synthesised by a supplier (for example MWG or Sigma-Genosys), so that each pair has the ability to hybridize to the opposite strands (3' ends pointing "towards" each other) flanking the stretch of *Clostridium* target DNA, one oligonucleotide for each of the two DNA strands. To generate a PCR product the pair of short oligonucleotide primers specific for the *Clostridium* DNA sequence are mixed with the *Clostridium* DNA template and other reaction components and placed in a machine (the 'PCR machine') that can change the incubation temperature of the reaction tube automatically, cycling between approximately 94°C (for denaturation), 55°C (for oligonucleotide annealing), and 72°C (for synthesis). Other reagents required for amplification of a PCR product include a DNA polymerase (such as *Taq* or *Pfu* polymerase), each of the four nucleotide dNTP building blocks of DNA in equimolar amounts (50-200 µM) and a buffer appropriate for the enzyme optimised for Mg^{2+} concentration (0.5-5 mM).

The amplification product is cloned into pCR 4 using either, TOPO TA cloning for *Taq* PCR products or Zero Blunt TOPO cloning for *Pfu* PCR products (both kits commercially available from Invitrogen). The resultant clone is checked by sequencing. Any additional restriction sequences which are not compatible with the cloning system are then removed using site directed mutagenesis [for example, using Quickchange (Stratagene Inc.)].

Preparation of translocation (e.g. H_N) insert

The H_N/A (SEQ ID2) is created by one of two ways:

The DNA sequence is designed by back translation of the H_N/A amino acid sequence [obtained from freely available database sources such as GenBank (accession number P10845) or Swissprot (accession locus BXA1_CLOBO)] using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)]. A *Pst*I restriction sequence added to the N-terminus and *Xba*I-stop codon-*Hin*dIII to the C-terminus ensuring the correct reading frame is

maintained. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

The alternative method is to use PCR amplification from an existing DNA sequence with *Pst*I and *Xba*I-stop codon-*Hin*dIII restriction enzyme sequences incorporated into the 5' and 3' PCR primers respectively. The PCR amplification is performed as described above. The PCR product is inserted into pCR 4 vector and checked by sequencing. Any additional restriction sequences which are not compatible with the cloning system are then removed using site directed mutagenesis [for example using Quickchange (Stratagene Inc.)].

Example 2 – Preparation of a LC/A-nociceptin-H_N/A fusion protein (nociceptin is N-terminal of the H_N-chain)

Preparation of linker-nociceptin-spacer insert

The LC-H_N linker can be designed from first principle, using the existing sequence information for the linker as the template. For example, the serotype A linker (in this case defined as the inter-domain polypeptide region that exists between the cysteines of the disulphide bridge between LC and H_N) is 23 amino acids long and has the sequence VRGIITSKTKSLDKGYNKALNDL. Within this sequence, it is understood that proteolytic activation in nature leads to an H_N domain that has an N-terminus of the sequence ALNDL. This sequence information is freely available from available database sources such as GenBank (accession number

P10845) or Swissprot (accession locus BXA1_CLOBO). Into this linker a Factor Xa site, nociceptin and spacer are incorporated; and using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)], the DNA sequence encoding the linker-ligand-spacer region is determined. Restriction sites are then incorporated into the DNA sequence and can be arranged as *Bam*HI-*Sal*I-linker-protease site-nociceptin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID7). It is important to ensure the correct reading frame is maintained for the spacer, nociceptin and restriction sequences and that the *Xba*I sequence is not preceded by the bases, TC, which would result on DAM methylation. The DNA sequence is screened for restriction sequence incorporation, and any additional sequences are removed manually from the remaining sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example, GenBank Release 143, 13 September 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

Preparation of the LC/A-nociceptin-H_N/A fusion

In order to create the LC-linker-nociceptin-spacer-H_N construct (SEQ ID13), the pCR 4 vector encoding the linker (SEQ ID7) is cleaved with *Bam*HI + *Sal*I restriction enzymes. This cleaved vector then serves as the recipient vector for insertion and ligation of the LC/A DNA (SEQ ID1) cleaved with *Bam*HI + *Sal*I. The resulting plasmid DNA is then cleaved with *Pst*I + *Xba*I restriction enzymes and serves as the recipient vector for the insertion and ligation of the H_N/A DNA (SEQ ID2) cleaved with *Pst*I + *Xba*I. The final construct contains the LC-linker-nociceptin-spacer-H_N ORF (SEQ ID13) for transfer into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID14.

Example 3 – Preparation of a nociceptin-LC/A-H_N/A fusion protein (nociceptin is N-terminal of the LC-chain)

The LC/A-H_N/A backbone is constructed as described in Example 2 using the synthesised A serotype linker with the addition of a Factor Xa site for activation, arranged as *Bam*HI-*Sal*I-linker-protease site-linker-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID8). The LC/A-H_N/A backbone and the synthesised N-terminal presentation nociceptin insert (SEQ ID9) are cleaved with *Bam*HI + *Hin*dIII restriction enzymes, gel purified and ligated together to create a nociceptin-spacer-LC-linker-H_N. The ORF (SEQ ID15) is then cut out using restriction enzymes *Ava*I + *Xba*I for transfer into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID16.

Example 4 – Preparation of a LC/C-nociceptin-H_N/C fusion protein

Following the methods used in Examples 1 and 2, the LC/C (SEQ ID5) and H_N/C (SEQ ID6) are created and inserted into the C serotype linker arranged as *Bam*HI-*Sal*I-linker-protease site-nociceptin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID10). The final construct contains the LC-linker-nociceptin-spacer-H_N ORF (SEQ ID17) for expression as a protein of the sequence illustrated in SEQ ID18.

Example 5 - Preparation of a LC/C-nociceptin-H_N/C fusion protein with a serotype A activation sequence

Following the methods used in Examples 1 and 2, the LC/C (SEQ ID5) and H_N/C (SEQ ID6) are created and inserted into the A serotype linker arranged as *Bam*HI-*Sal*I-linker-protease site-nociceptin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID7). The final construct contains the LC-linker-nociceptin-spacer-H_N ORF (SEQ ID19) for expression as a protein of the sequence illustrated in SEQ ID20.

Example 6 - Preparation of a LC/A-met enkephalin-H_N/A fusion protein

Due to the small, five-amino acid, size of the met-enkephalin ligand the LC/A-met enkephalin-H_N/A fusion is created by site directed mutagenesis [for example using Quickchange (Stratagene Inc.)] using the LC/A-nociceptin-H_N/A fusion (SEQ ID13) as a template. Oligonucleotides are designed encoding the YGGFM met-enkephalin peptide, ensuring standard *E.coli* codon usage is maintained and no additional restriction sites are incorporated, flanked by sequences complimentary to the linker region of the LC/A-nociceptin-H_N/A fusion (SEQ ID13) either side on the nociceptin section. The SDM product is checked by sequencing and the final construct containing the LC-linker-met enkephalin-spacer-H_N ORF (SEQ ID21) for expression as a protein of the sequence illustrated in SEQ ID22.

Example 7 - Preparation of a LC/A-β endorphin-H_N/A fusion protein

Following the methods used in Examples 1 and 2, the LC/A (SEQ ID1) and H_N/A (SEQ ID2) are created and inserted into the A serotype β endorphin linker arranged as *Bam*HI-*Sal*I-linker-protease site-β endorphin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID11). The final construct contains the LC-linker-β endorphin-spacer-H_N ORF (SEQ ID23) for expression as a protein of the sequence illustrated in SEQ ID24.

Example 8 - Preparation of a LC/A-nociceptin variant-H_N/A fusion protein

Following the methods used in Examples 1 and 2, the LC/A (SEQ ID1) and H_N/A (SEQ ID2) are created and inserted into the A serotype nociceptin variant linker arranged as *Bam*HI-*Sal*I-linker-protease site-nociceptin variant-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID12). The final construct contains the LC-linker-nociceptin variant-spacer-H_N ORF (SEQ ID25) for expression as a protein of the sequence illustrated in SEQ ID26.

Example 9 – Purification method for LC/A-nociceptin-H_N/A fusion protein

Defrost falcon tube containing 25 ml 50 mM HEPES pH 7.2, 200 mM NaCl and approximately 10 g of *E. coli* BL21 cell paste. Make the thawed cell paste up-to 80 ml with 50 mM HEPES pH 7.2, 200 mM NaCl and sonicate on ice 30 seconds on, 30 seconds off for 10 cycles at a power of 22 microns ensuring the sample remains cool. Spin the lysed cells at 18 000 rpm, 4°C for 30 minutes. Load the supernatant onto a 0.1 M $NiSO_4$ charged Chelating column (20-30 ml column is sufficient) equilibrated with 50 mM HEPES pH 7.2, 200 mM NaCl. Using a step gradient of 10 and 40 mM imidazol, wash away the non-specific bound protein and elute the fusion protein with 100 mM imidazol. Dialyse the eluted fusion protein against 5 L of 50 mM HEPES pH 7.2, 200 mM NaCl at 4°C overnight and measure the OD of the dialysed fusion protein. Add 1 unit of factor Xa per 100 µg fusion protein and Incubate at 25°C static overnight. Load onto a 0.1 M $NiSO_4$ charged Chelating column (20-30 ml column is sufficient) equilibrated with 50 mM HEPES pH 7.2, 200 mM NaCl. Wash column to baseline with 50 mM HEPES pH 7.2, 200 mM NaCl. Using a step gradient of 10 and 40 mM imidazol, wash away the non-specific bound protein and elute the fusion protein with 100 mM imidazol. Dialyse the eluted fusion protein against 5 L of 50 mM HEPES pH 7.2, 200 mM NaCl at 4°C overnight and concentrate the fusion to about 2 mg/ml, aliquot sample and freeze at -20°C. Test purified protein using OD, BCA, purity analysis and SNAP-25 assessments.

Example 10 – Confirmation of TM Agonist Activity by measuring release of substance P from neuronal cell cultures

Materials

Substance P EIA is obtained from R&D Systems, UK.

Methods

Primary neuronal cultures of eDRG are established as described previously (Duggan *et al.*, 2002). Substance P release from the cultures is assessed by EIA, essentially as described previously (Duggan *et al.*, 2002). The TM of interest is added to the neuronal cultures (established for at least 2 weeks prior to treatment); control cultures are performed in parallel by addition of vehicle in place of TM. Stimulated (100 mM KCl) and basal release, together with total cell lysate content, of substance P are obtained for both control and TM treated cultures. Substance P immunoreactivity is measured using Substance P Enzyme Immunoassay Kits (Cayman Chemical Company, USA or R&D Systems, UK) according to manufacturers' instructions.

The amount of Substance P released by the neuronal cells in the presence of the TM of interest is compared to the release obtained in the presence and absence of 100 mM KCl. Stimulation of Substance P release by the TM of interest above the basal release, establishes that the TM of interest is an "agonist ligand" as defined in this specification. If desired the stimulation of Substance P release by the TM of interest can be compared to a standard Substance P release-curve produced using the natural ORL-1 receptor ligand, nociceptin (Tocris).

Example 11 - Confirmation of ORL_1 receptor activation by measuring forskolin-stimulated cAMP production

Confirmation that a given TM is acting via the ORL_1 receptor is provided by the following test, in which the TMs ability to inhibit forskolin-stimulated cAMP production is assessed.

Materials

[^{3}H]adenine and [^{14}C]cAMP are obtained from GE Healthcare

Methods

The test is conducted essentially as described previously by Meunier *et al.* [Isolation and structure of the endogenous agonist of opioid receptor-like ORL_1

receptor. Nature 377: 532-535, 1995] in intact transfected-CHO cells plated on 24-well plastic plates.

To the cells is added [3H]adenine (1.0 μCi) in 0.4 ml of culture medium. The cells remain at 37°C for 2 h to allow the adenine to incorporate into the intracellular ATP pool. After 2 h, the cells are washed once with incubation buffer containing: 130 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 1 mg/ml bovine serum albumin and 25 mM HEPES pH 7.4, and replaced with buffer containing forskolin (10 μM) and isobutylmethylxanthine (50 μM) with or without the TM of interest. After 10 min, the medium is aspirated and replaced with 0.5 ml, 0.2 M HCl. Approximately 1000 cpm of [^{14}C]cAMP is added to each well and used as an internal standard. The contents of the wells are then transferred to columns of 0.65 g dry alumina powder. The columns are eluted with 4 ml of 5 mM HCl, 0.5 ml of 0.1 M ammonium acetate, then two additional millilitres of ammonium acetate. The final eluate is collected into scintillation vials and counted for ^{14}C and tritium. Amounts collected are corrected for recovery of [^{14}C]cAMP. TMs that are agonists at the ORL_1 receptor cause a reduction in the level of cAMP produced in response to forskolin.

Example 12 - Confirmation of ORL_1 receptor activation using a GTPγS binding functional assay

Confirmation that a given TM is acting via the ORL_1 receptor is also provided by the following test, a GTPγS binding functional assay.

Materials

[^{35}S]GTPγS is obtained from GE Healthcare

Wheatgerm agglutinin-coated (SPA) beads are obtained from GE Healthcare

Methods

This assay is carried out essentially as described by Traynor and Nahorski [Modulation by μ-opioid agonists of guanosine-5 -O-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. Mol. Pharmacol. 47: 848-854, 1995].

Cells are scraped from tissue culture dishes into 20 mM HEPES, 1 mM ethylenediaminetetraacetic acid, then centrifuged at 500 × *g* for 10 min. Cells are resuspended in this buffer and homogenized with a Polytron Homogenizer.

The homogenate is centrifuged at 27,000 × *g* for 15 min, and the pellet resuspended in buffer A, containing: 20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4. The suspension is recentrifuged at 20,000 × *g* and suspended once more in buffer A. For the binding assay, membranes (8-15 μg protein) are incubated with [^{35}S]GTP S (50 pM), GDP (10 μM), with and without the TM of interest, in a total volume of 1.0 ml, for 60 min at 25°C. Samples are filtered over glass fibre filters and counted as described for the binding assays.

Example 13 – Preparation of a LC/A-nociceptin-H_N/A fusion protein (nociceptin is N-terminal of the H_N-chain)

The linker-nociceptin-spacer insert is prepared as described in Example 2.

Preparation of the LC/A-nociceptin-H_N/A fusion

In order to create the LC-linker-nociceptin-spacer-H_N construct (SEQ ID13), the pCR 4 vector encoding the linker (SEQ ID7) is cleaved with *Bam*HI + *Sal*I restriction enzymes. This cleaved vector then serves as the recipient for insertion and ligation of the LC/A DNA (SEQ ID1) also cleaved with *Bam*HI + *Sal*I. The resulting plasmid DNA is then cleaved with *Bam*HI + *Hind*III restriction enzymes and the LC/A-linker fragment inserted into a similarly cleaved vector containing a unique multiple cloning site for *Bam*HI, *Sal*I, *Pst*I, and *Hind*III such as the pMAL

vector (NEB). The H_N/A DNA (SEQ ID2) is then cleaved with *Pst*I + *Hind*III restriction enzymes and inserted into the similarly cleaved pMAL-LC/A-linker construct. The final construct contains the LC-linker-nociceptin-spacer-H_N ORF (SEQ ID13) for expression as a protein of the sequence illustrated in SEQ ID14.

Example 14 – Preparation of a nociceptin-LC/A-H_N/A fusion protein (nociceptin is N-terminal of the LC-chain)

In order to create the nociceptin-spacer-LC/A-H_N/A construct, an A serotype linker with the addition of a Factor Xa site for activation, arranged as *Bam*HI-*Sal*I-linker-protease site-linker-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID8) is synthesised as described in Example 13. The pCR 4 vector encoding the linker is cleaved with *Bam*HI + *Sal*I restriction enzymes. This cleaved vector then serves as the recipient for insertion and ligation of the LC/A DNA (SEQ ID1) also cleaved with *Bam*HI + *Sal*I. The resulting plasmid DNA is then cleaved with *Bam*HI + *Hind*III restriction enzymes and the LC/A-linker fragment inserted into a similarly cleaved vector containing the synthesised N-terminal presentation nociceptin insert (SEQ ID9). This construct is then cleaved with *Ava*I + *Hind*III and inserted into an expression vector such as the pMAL plasmid (NEB). The H_N/A DNA (SEQ ID2) is then cleaved with *Pst*I + *Hind*III restriction enzymes and inserted into the similarly cleaved pMAL-nociceptin-LC/A-linker construct. The final construct contains the nociceptin-spacer-LC/A-H_N/A ORF (SEQ ID51) for expression as a protein of the sequence illustrated in SEQ ID52.

Example 15 - Preparation and purification of an LC/A-nociceptin-H_N/A fusion protein family with variable spacer length

Using the same strategy as employed in Example 2, a range of DNA linkers were prepared that encoded nociceptin and variable spacer content. Using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)], the DNA sequence encoding the linker-ligand-spacer region is determined.

Restriction sites are then incorporated into the DNA sequence and can be arranged as *Bam*HI-*Sal*I-linker-protease site-nociceptin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID53 to SEQ ID57). It is important to ensure the correct reading frame is maintained for the spacer, nociceptin and restriction sequences and that the *Xba*I sequence is not preceded by the bases, TC which would result on DAM methylation. The DNA sequence is screened for restriction sequence incorporation and any additional sequences are removed manually from the remaining sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

The spacers that were created included:

Code	Protein sequence of the linker	SEQ ID of the linker DNA
GS10	ALAGGGGSALVLQ	53
GS15	ALAGGGGSGGGGSALVLQ	54
GS25	ALAGGGGSGGGGSGGGGSGGGGSALVLQ	55
GS30	ALAGGGGSGGGGSGGGGSGGGGSGGGGSALVLQ	56
HX27	ALAAEAAAKEAAAKEAAAKAGGGGSALVLQ	57

Table 1

By way of example, in order to create the LC/A-CPN(GS15)-H_N/A fusion construct (SEQ ID58), the pCR 4 vector encoding the linker (SEQ ID54) is cleaved with *Bam*HI + *Sal*I restriction enzymes. This cleaved vector then serves as the recipient vector for insertion and ligation of the LC/A DNA (SEQ ID1) also cleaved with *Bam*HI + *Sal*I. The resulting plasmid DNA is then cleaved with *Bam*HI + *Hin*dIII restriction enzymes and the LC/A-linker fragment inserted into a similarly

cleaved vector containing a unique multiple cloning site for *Bam*HI, *Sal*I, *Pst*I, and *Hind*III such as the pMAL vector (NEB). The H_N/A DNA (SEQ ID2) is then cleaved with *Pst*I + *Hind*III restriction enzymes and inserted into the similarly cleaved pMAL-LC/A-linker construct. The final construct contains the LC/A-CPN(GS15)-H_N/A ORF (SEQ ID58) for expression as a protein of the sequence illustrated in SEQ ID59.

As a further example, to create the LC/A-CPN(GS25)-H_N/A fusion construct (SEQ ID60), the pCR 4 vector encoding the linker (SEQ ID55) is cleaved with *Bam*HI + *Sal*I restriction enzymes. This cleaved vector then serves as the recipient vector for insertion and ligation of the LC/A DNA (SEQ ID1) cleaved with *Bam*HI + *Sal*I. The resulting plasmid DNA is then cleaved with *Bam*HI + *Hind*III restriction enzymes and the LC/A-linker fragment inserted into a similarly cleaved vector containing a unique multiple cloning site for *Bam*HI, *Sal*I, *Pst*I, and *Hind*III such as the pMAL vector (NEB). The H_N/A DNA (SEQ ID2) is then cleaved with *Pst*I + *Hind*III restriction enzymes and inserted into the similarly cleaved pMAL-LC/A-linker construct. The final construct contains the LC/A-CPN(GS25)-H_N/A ORF (SEQ ID60) for expression as a protein of the sequence illustrated in SEQ ID61.

Variants of the LC/A-CPN-H_N/A fusion consisting of GS10, GS30 and HX27 are similarly created. Using the purification methodology described in Example 9, fusion protein is purified from *E. coli* cell paste. Figure 9 illustrates the purified product obtained in the case of LC/A-CPN(GS10)-H_N/A, LC/A-CPN(GS15)-H_N/A, LC/A-CPN(GS25)-H_N/A, LC/A-CPN(GS30)-H_N/A and LC/A-CPN(HX27)-H_N/A.

Example 16 - Assessment of *in vitro* efficacy of an LC/A-nociceptin-H_N/A fusion

Fusion protein prepared according to Examples 2 and 9 was assessed in the eDRG neuronal cell model.

Assays for the inhibition of substance P release and cleavage of SNAP-25 have been previously reported (Duggan *et al.*, 2002, *J. Biol. Chem.*, 277, 34846-34852). Briefly, dorsal root ganglia neurons are harvested from 15-day-old fetal Sprague-Dawley rats and dissociated cells plated onto 24-well plates coated with Matrigel at a density of 1 x 10^6 cells/well. One day post-plating the cells are treated with 10 μM cytosine β-D-arabinofuranoside for 48 h. Cells are maintained in Dulbecco's minimal essential medium supplemented with 5% heat-inactivated fetal bovine serum, 5 mM L-glutamine, 0.6% D-glucose, 2% B27 supplement, and 100 ng/ml 2.5S mouse nerve growth factor. Cultures are maintained for 2 weeks at 37°C in 95% air/5% CO_2 before addition of test materials.

Release of substance P from eDRG is assessed by enzyme-linked immunosorbent assay. Briefly, eDRG cells are washed twice with low potassium-balanced salt solution (BSS: 5 mM KCl, 137 mM NaCl, 1.2 mM $MgCl_2$, 5 mM glucose, 0.44 mM KH_2PO_4, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$). Basal samples are obtained by incubating each well for 5 min. with 1 ml of low potassium BSS. After removal of this buffer, the cells are stimulated to release by incubation with 1 ml of high potassium buffer (BSS as above with modification to include 100 mM KCl isotonically balanced with NaCl) for 5 min. All samples are removed to tubes on ice prior to assay of substance P. Total cell lysates are prepared by addition of 250 μl of 2 M acetic acid/0.1% trifluoroacetic acid to lyse the cells, centrifugal evaporation, and resuspension in 500 μl of assay buffer. Diluted samples are assessed for substance P content. Substance P immunoreactivity is measured using Substance P Enzyme Immunoassay Kits (Cayman Chemical Company or R&D Systems) according to manufacturers' instructions. Substance P is expressed in pg/ml relative to a standard substance P curve run in parallel.

SDS-PAGE and Western blot analysis were performed using standard protocols (Novex). SNAP-25 proteins were resolved on a 12% Tris/glycine polyacrylamide gel (Novex) and subsequently transferred to nitrocellulose membrane. The

membranes were probed with a monoclonal antibody (SMI-81) that recognises cleaved and intact SNAP-25. Specific binding was visualised using peroxidase-conjugated secondary antibodies and a chemiluminescent detection system. Cleavage of SNAP-25 was quantified by scanning densitometry (Molecular Dynamics Personal SI, ImageQuant data analysis software). Percent SNAP-25 cleavage was calculated according to the formula: (Cleaved SNAP-25/(Cleaved+Intact SNAP-25))x100.

Following exposure of eDRG neurons to an LC/A-nociceptin-H_N/A fusion (termed CPN-A), both inhibition of substance P release and cleavage of SNAP-25 are observed (Figure 10). After 24 h exposure to the fusion, 50% of maximal SNAP-25 cleavage is achieved by a fusion concentration of 6.3±2.5 nM.

The effect of the fusion is also assessed at defined time points following a 16 h exposure of eDRG to CPN-A. Figure 11 illustrates the prolonged duration of action of the CPN-A fusion protein, with measurable activity still being observed at 28 days post exposure.

Example 17 - Assessment of *in vitro* efficacy of an LC/A-nociceptin variant-H_N/A fusion

Fusion protein prepared according to Examples 8 and 9 was assessed in the eDRG neuronal cell mode using the method described in Example 16.

Following exposure of eDRG neurons to an LC/A-nociceptin variant-H_N/A fusion (termed CPNv-A), both inhibition of substance P release and cleavage of SNAP-25 are observed. After 24 h exposure to the fusion, 50% of maximal SNAP-25 cleavage is achieved by a fusion concentration of 1.4±0.4 nM (Figure 12).

The effect of the fusion is also assessed at defined time points following a 16 h exposure of eDRG to CPN-A. Figure 13 illustrates the prolonged duration of

action of the CPN-A fusion protein, with measurable activity still being observed at 24 days post exposure.

The binding capability of the CPNv-A fusion protein is also assessed in comparison to the CPN-A fusion. Figure 14 illustrates the results of a competition experiment to determine binding efficacy at the ORL-1 receptor. CPNv-A is demonstrated to displace [3H]-nociceptin, thereby confirming that access to the receptor is possible with the ligand in the central presentation format.

Example 18 - Preparation of an LC/A-nociceptin variant-H_N/A fusion protein that is activated by treatment with Enterokinase

Following the methods used in Examples 1 and 2, the LC/A (SEQ ID1) and H_N/A (SEQ ID2) are created and inserted into the A serotype nociceptin variant linker arranged as *Bam*HI-*Sal*I-linker-enterokinase protease site-nociceptin variant-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID62). The final construct contains the LC-linker-nociceptin variant-spacer-H_N ORF sequences (SEQ ID63) for expression as a protein of the sequence illustrated in SEQ ID64. The fusion protein is termed CPNv(Ek)-A. Figure 15 illustrates the purification of CPNv(Ek)-A from *E. coli* following the methods used in Example 9 but using Enterokinase for activation at 0.00064 µg per 100 µg of fusion protein.

Example 19 - Assessment of *in vitro* efficacy of a LC/A-nociceptin variant-H_N/A fusion that has been activated by treatment with enterokinase

The CPNv(Ek)-A prepared in Example 18 is obtained in a purified form and applied to the eDRG cell model to assess cleavage of SNAP-25 (using methodology from Example 16). Figure 16 illustrates the cleavage of SNAP-25 following 24 h exposure of eDRG to CPNv(Ek)-A. The efficiency of cleavage is observed to be similar to that achieved with the Factor Xa-cleaved material, as recorded in Example 17.

Example 20 - Preparation of an LC/C-nociceptin variant-H_N/C fusion protein with a Factor Xa activation linker derived from serotype A

Following the methods used in Example 4, the LC/C (SEQ ID5) and H_N/C (SEQ ID6) are created and inserted into the A serotype nociceptin variant linker arranged as *Bam*HI-*Sal*I-linker-nociceptin variant-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID65). The final construct contains the LC-linker-nociceptin variant-spacer-H_N ORF sequences (SEQ ID66) for expression as a protein of the sequence illustrated in SEQ ID67. The fusion protein is termed CPNv-C (act. A). Figure 17 illustrates the purification of CPNv-C (act. A) from *E. coli* following the methods used in Example 9.

Example 21 - Assessment of *in vitro* efficacy of an LC/C-nociceptin variant-H_N/C fusion protein

Following the methods used in Example 9, the CPNv-C (act. A) prepared in Example 20 is obtained in a purified form and applied to the eDRG cell model to assess cleavage of SNAP-25 (using methodology from Example 16). After 24 h exposure to the fusion, 50% of maximal syntaxin cleavage is achieved by a fusion concentration of 3.1±2.0 nM. Figure 18 illustrates the cleavage of syntaxin following 24 h exposure of eDRG to CPNv-C (act. A).

Example 22 - Assessment of *in vivo* efficacy of an LC/A-nociceptin-HN/A fusion

The ability of an LC/A-nociceptin- H_N/A fusion (CPN/A) to inhibit acute capsaicin-induced mechanical allodynia is evaluated following subcutaneous intraplantar injection in the rat hind paw. Test animals are evaluated for paw withdrawal frequency (PWF%) in response to a 10 g Von Frey filament stimulus series (10 stimuli x 3 trials) prior to recruitment into the study, after subcutaneous treatment with CPN/A but before capsaicin, and following capsaicin challenge post-injection of CPN/A (average of responses at 15' and 30'). Capsaicin challenge is achieved

by injection of 10 µL of a 0.3% solution. Sample dilutions are prepared in 0.5% BSA/saline. Figure 19 illustrates the reversal of mechanical allodynia that is achieved by pre-treatment of the animals with a range of concentrations of LC/A-nociceptin-HN/A fusion.

The ability of an LC/A-nociceptin-HN/A fusion (CPN/A) to inhibit streptozotocin (STZ)– induced mechanical (tactile) allodynia in rats is evaluated. STZ–induced mechanical allodynia in rats is achieved by injection of streptozotocin (i.p. or i.v.) which yields destruction of pancreatic β-cells leading to loss of insulin production, with concomitant metabolic stress (hyperglycemia and hyperlipidemia). As such, STZ induces Type I diabetes. In addition, STZ treatment leads to progressive development of neuropathy, which serves as a model of chronic pain with hyperalgesia and allodynia that may reflect signs observed in diabetic humans (peripheral diabetic neuropathy).

Male Sprague-Dawley rats (250-300 g) are treated with 65 mg/kg STZ in citrate buffer (I.V.) and blood glucose and lipid are measured weekly to define the readiness of the model. Paw Withdrawal Threshold (PWT) is measured in response to a Von Frey filament stimulus series over a period of time. Allodynia is said to be established when the PWT on two consecutive test dates (separated by 1 week) measures below 6 g on the scale. At this point, rats are randomized to either a saline group (negative efficacy control), gabapentin group (positive efficacy control) or a test group (CPN/A). Test materials (20-25 µl) are injected subcutaneously as a single injection (except gabapentin) and the PWT is measured at 1 day post-treatment and periodically thereafter over a 2-week period. Gabapentin (30 mg/kg i.p. @ 3 ml/kg injection volume) is injected daily, 2 hours prior to the start of PWT testing. Figure 20 illustrates the reversal of allodynia achieved by pre-treatment of the animals with 750 ng of CPN/A. Data were obtained over a 2-week period after a single injection of CPN/A

Example 23 - Assessment of *in vivo* efficacy of an LC/A-nociceptin variant-H_N/A fusion

The ability of an LC/A-nociceptin variant-H_N/A fusion (CPNv/A) to inhibit capsaicin-induced mechanical allodynia is evaluated following subcutaneous intraplantar injection in the rat hind paw. Test animals are evaluated for paw withdrawal frequency (PWF%) in response to a 10 g Von Frey filament stimulus series (10 stimuli x 3 trials) prior to recruitment into the study (Pre-Treat); after subcutaneous intraplantar treatment with CPNv/A but before capsaicin (Pre-CAP); and following capsaicin challenge post-injection of CPNv/A (average of responses at 15' and 30'; CAP). Capsaicin challenge is achieved by injection of 10 µL of a 0.3% solution. Sample dilutions are prepared in 0.5% BSA/saline.

Figure 21 illustrates the reversal of allodynia that is achieved by pre-treatment of the animals with a range of concentrations of LC/A-nociceptin variant-H_N/A fusion in comparison to the reversal achieved with the addition of LC/A-nociceptin-H_N/A fusion. These data are expressed as a normalized paw withdrawal frequency differential, in which the difference between the peak response (post-capsaicin) and the baseline response (pre-capsaicin) is expressed as a percentage. With this analysis, it can be seen that CPNv/A is more potent than CPN/A since a lower dose of CPNv/A is required to achieve similar analgesic effect to that seen with CPN/A.

Example 24 - Preparation of an LC/A-leu enkephalin-H_N/A fusion protein

Due to the small, five-amino acid, size of the leu-enkephalin ligand the LC/A-leu enkephalin-H_N/A fusion is created by site directed mutagenesis [for example using Quickchange (Stratagene Inc.)] using the LC/A-nociceptin-H_N/A fusion (SEQ ID13) as a template. Oligonucleotides are designed encoding the YGGFL leu-enkephalin peptide, ensuring standard *E. coli* codon usage is maintained and no additional restriction sites are incorporated, flanked by sequences complimentary to the linker region of the LC/A-nociceptin-H_N/A fusion (SEQ ID13) either side on the nociceptin section. The SDM product is checked by sequencing and the final construct containing the LC-linker-leu enkephalin-

spacer-H_N ORF (SEQ ID68) for expression as a protein of the sequence illustrated in SEQ ID69. The fusion protein is termed CPLE-A. Figure 22 illustrates the purification of CPLE-A from *E. coli* following the methods used in Example 9.

Example 25 – Expression and purification of an LC/A-beta-endorphin-H_N/A fusion protein

Following the methods used in Example 9, and with the LC/A-beta-endorphin-H_N/A fusion protein (termed CPBE-A) created in Example 7, the CPBE-A is purified from *E. coli*. Figure 23 illustrates the purified protein as analysed by SDS-PAGE.

Example 26 - Preparation of an LC/A-nociceptin mutant-H_N/A fusion protein

Due to the single amino acid modification necessary to mutate the nociceptin sequence at position 1 from a Phe to a Tyr, the LC/A-nociceptin mutant-H_N/A fusion is created by site directed mutagenesis [for example using Quickchange (Stratagene Inc.)] using the LC/A-nociceptin-H_N/A fusion (SEQ ID13) as a template. Oligonucleotides are designed encoding tyrosine at position 1 of the nociceptin sequence, ensuring standard *E. coli* codon usage is maintained and no additional restriction sites are incorporated, flanked by sequences complimentary to the linker region of the LC/A-nociceptin-H_N/A fusion (SEQ ID13) either side on the nociceptin section. The SDM product is checked by sequencing and the final construct containing the LC/A-nociceptin mutant-spacer-H_N/A fusion ORF (SEQ ID70) for expression as a protein of the sequence illustrated in SEQ ID71. The fusion protein is termed CPOP-A. Figure 24 illustrates the purification of CPOP-A from *E. coli* following the methods used in Example 9.

Example 27 - Preparation and assessment of an LC/A-nociceptin variant mutant-H_N/A fusion protein

Due to the single amino acid modification necessary to mutate the nociceptin sequence at position 1 from a Phe to a Tyr, the LC/A-nociceptin variant mutant-H_N/A fusion is created by site directed mutagenesis [for example using Quickchange (Stratagene Inc.)] using the LC/A-nociceptin variant-H_N/A fusion (SEQ ID25) as a template. Oligonucleotides are designed encoding tyrosine at position 1 of the nociceptin sequence, ensuring standard *E. coli* codon usage is maintained and no additional restriction sites are incorporated, flanked by sequences complimentary to the linker region of the LC/A-nociceptin variant-H_N/A fusion (SEQ ID25) either side on the nociceptin section. The SDM product is checked by sequencing and the final construct containing the LC/A-nociceptin mutant-spacer-H_N/A fusion ORF (SEQ ID72) for expression as a protein of the sequence illustrated in SEQ ID73. The fusion protein is termed CPOPv-A. Figure 25 illustrates the purification of CPOPv-A from *E. coli* following the methods used in Example 9.

Using methodology described in Example 16, CPOPv-A is assessed for its ability to cleave SNAP-25 in the eDRG cell model. Figure 26 illustrates that CPOPv-A is able to cleave SNAP-25 in the eDRG model, achieving cleavage of 50% of the maximal SNAP-25 after exposure of the cells to approximately 5.9 nM fusion for 24 h.

Example 28 - Preparation of an IgA protease-nociceptin variant-H_N/A fusion protein

The IgA protease amino acid sequence was obtained from freely available database sources such as GenBank (accession number P09790). Information regarding the structure of the *N. Gonorrhoeae* IgA protease gene is available in the literature (Pohlner *et al.*, Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease, *Nature*, 1987, 325(6103), 458-62). Using Backtranslation tool v2.0 (Entelechon), the DNA sequence encoding the IgA protease modified for *E. coli* expression was determined. A *Bam*HI recognition

sequence was incorporated at the 5' end and a codon encoding a cysteine amino acid and *Sal*I recognition sequence were incorporated at the 3' end of the IgA DNA. The DNA sequence was screened using MapDraw, (DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required for cloning were removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage was assessed Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables. This optimised DNA sequence (SEQ ID74) containing the IgA open reading frame (ORF) is then commercially synthesized.

The IgA (SEQ ID74) is inserted into the LC-linker-nociceptin variant-spacer-H_N ORF (SEQ ID25) using *Bam*HI and *Sal*I restriction enzymes to replace the LC with the IgA protease DNA. The final construct contains the IgA-linker-nociceptin variant-spacer-H_N ORF (SEQ ID75) for expression as a protein of the sequence illustrated in SEQ ID76.

Example 29 - Preparation and assessment of a nociceptin targeted endopeptidase fusion protein with a removable histidine purification tag.

DNA was prepared that encoded a Factor Xa removable his-tag (his6), although it is clear that alternative proteases site such as Enterokinase and alternative purification tags such as longer histidine tags are also possible. Using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)], the DNA sequence encoding the Factor Xa removable his-tag region is determined. Restriction sites are then incorporated into the DNA sequence and can be arranged as *Nhe*I-linker-*Spe*I-*Pst*I-H_N/*A*-*Xba*I-*LEIEGRSGHHHHHHStop codon-Hind*III (SEQ ID77). The DNA sequence is screened for restriction sequence incorporated and any additional sequences are removed manually from the remaining sequence ensuring common *E. coli* codon usage is

maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector. In order to create CPNv-A-FXa-HT (SEQ ID78, removable his-tag construct) the pCR 4 vector encoding the removable his-tag is cleaved with *Nhe*I and *Hind*III. The *Nhe*I - *Hind*III fragment is then inserted into the LC/A-CPNv-H_N/A vector (SEQ ID25) that has also been cleaved by *Nhe*I and *Hind*III. The final construct contains the LC/A-linker-nociceptin variant-spacer-H_N-FXa-Histag-*Hind*III ORF sequences (SEQ ID78) for expression as a protein of the sequence illustrated in SEQ ID79. Figure 27 illustrates the purification of CPNv-A-FXa-HT from *E. coli* following the methods used in Example 9.

Example 30 - Preparation of a leu-enkephalin targeted endopeptidase fusion protein containing a translocation domain derived from diphtheria toxin

The DNA sequence is designed by back translation of the amino acid sequence of the translocation domain of the diphtheria toxin (obtained from freely available database sources such as GenBank (accession number 1XDTT) using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)]. Restriction sites are then incorporated into the DNA sequence and can be arranged as *Nhe*I-Linker-*Spe*I-*Pst*I- diphtheria translocation domain-*Xba*I-stop codon-*Hind*III (SEQ ID80). *PstI*/*Xba*I recognition sequences are incorporated at the 5' and 3' ends of the translocation domain respectively of the sequence maintaining the correct reading frame. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common *E. coli*

codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence containing the diphtheria translocation domain is then commercially synthesized as *Nhe*I-Linker-*Spe*I-*Pst*I- diphtheria translocation domain-*Xba*I-stop codon-*Hind*III (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector (Invitrogen). The pCR 4 vector encoding the diphtheria translocation domain is cleaved with *Nhe*I and *Xba*I. The *Nhe*I – *Xba*I fragment is then inserted into the LC/A-CPLE-H_N/A vector (SEQ ID68) that has also been cleaved by *Nhe*I and *Xba*I. The final construct contains the LC/A-leu-enkephalin-spacer-diphtheria translocation domain ORF sequences (SEQ ID81) for expression as a protein of the sequence illustrated in SEQ ID82.

Example 31 - Preparation of a nociceptin variant targeted endopeptidase fusion protein containing a LC domain derived from tetanus toxin.

The DNA sequence is designed by back translation of the tetanus toxin LC amino acid sequence (obtained from freely available database sources such as GenBank (accession number X04436) using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)]. *Bam*HI/*Sal*I recognition sequences are incorporated at the 5' and 3' ends respectively of the sequence maintaining the correct reading frame (SEQ ID83). The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143,

13 September 2004). This optimised DNA sequence containing the tetanus toxin LC open reading frame (ORF) is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector (invitrogen). The pCR 4 vector encoding the TeNT LC is cleaved with B*am*HI and *Sal*I. The *Bam*HI – *Sal*I fragment is then inserted into the LC/A-CPNv-H_N/A vector (SEQ ID25) that has also been cleaved by *Bam*HI and *Sal*I. The final construct contains the TeNT LC-linker-nociceptin variant-spacer-H_N ORF sequences (SEQ ID84) for expression as a protein of the sequence illustrated in SEQ ID85.

Example 32 - Preparation of an LC/C-nociceptin variant-H_N/C fusion protein with a native serotype C linker that is susceptible to Factor Xa cleavage

Following the methods used in Example 4, the LC/C (SEQ ID5) and H_N/C (SEQ ID6) are created and inserted into the C serotype nociceptin variant linker arranged as *Bam*HI-*Sal*I-linker-nociceptin variant-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hin*dIII (SEQ ID86). The final construct contains the LC-linker-nociceptin variant-spacer-H_N ORF sequences (SEQ ID87) for expression as a protein of the sequence illustrated in SEQ ID88. The fusion protein is termed CPNv-C (act. C).

Claims:

1. Use of a therapeutic molecule for the manufacture of a medicament for the treatment of particular types of pain, wherein the therapeutic molecule is a single chain, polypeptide fusion protein, comprising:

   a. a non-cytotoxic protease, or a fragment thereof, which protease or protease fragment is capable of cleaving a protein of the exocytic fusion apparatus of a nociceptive sensory afferent;

   b. a Targeting Moiety that is capable of binding to a Binding Site on the nociceptive sensory afferent, which Binding Site is capable of undergoing endocytosis to be incorporated into an endosome within the nociceptive sensory afferent;

   c. a protease cleavage site at which site the fusion protein is cleavable by a protease, wherein the protease cleavage site is located between the non-cytotoxic protease or fragment thereof and the Targeting Moiety; and

   d. a translocation domain that is capable of translocating the protease or protease fragment from within an endosome, across the endosomal membrane and into the cytosol of the nociceptive sensory afferent.

2. Use according to Claim 1, wherein the Targeting Moiety and the protease cleavage site are separated by at most 10 amino acid residues, preferably by at most 5 amino acid residues, and more preferably by at most zero amino acid residues.

3. Use according to Claim 1 or Claim 2, wherein the Targeting Moiety is located between the protease cleavage site and the translocation domain.

4. Use according to any preceding claim, wherein the non-cytotoxic protease is a clostridial neurotoxin L-chain or an IgA protease.

5. Use according to any preceding claim, wherein the translocation domain is the H_N domain of a clostridial neurotoxin.

6. Use according to any preceding claim, wherein the Targeting Moiety comprises at most 50 amino acid residues, preferably at most 40 amino acid residues, more preferably at least 30 amino acid residues, and most preferably at most 20 amino acid residues.

7. Use according to any of Claims 1-6, wherein the Targeting Moiety is an opioid.

8. Use according to any of Claim 1-6, wherein the Targeting Moiety is an agonist of a receptor present on a nociceptive sensory afferent.

9. Use according to Claim 8, wherein the Targeting Moiety is an agonist of a receptor present on a primary nociceptive sensory afferent.

10. Use according to any of Claims 1-6, wherein the Targeting Moiety binds to the ORL_1 receptor.

11. Use according to Claim 10, wherein the Targeting Moiety binds specifically to the ORL_1 receptor.

12. Use according to Claim 10 or 11, wherein the Targeting Moiety is an agonist of the ORL_1 receptor.

13. Use according to any one of Claims 10-12, wherein the Targeting Moiety has at least 70% homology to SEQ ID No. 38 or a fragment thereof.

14. Use according to Claim 13, wherein the Targeting Moiety as at least 80% homology to SEQ ID No. 38 or a fragment thereof.

15. Use according to Claim 14, wherein the Targeting Moiety has at least 90% homology to SEQ ID No. 38 or a fragment thereof.

16. Use according to Claim 15, wherein the Targeting Moiety has at least 95% homology to SEQ ID No. 38 or a fragment thereof.

17. Use according to any one of Claims 10-12, wherein the Targeting Moiety is SEQ ID No. 38 or a fragment thereof.

18. Use according to any of Claims 10-12, wherein the Targeting Moiety is one of SEQ ID Nos: 40, 42, 44, 46, 48 or 50.

19. Use according to any one of Claims 10-12, wherein the Targeting Moiety is nociceptin.

20. Use according to any of Claims 1-6, wherein the Targeting Moiety is selected from the group consisting of nociceptin, β-endorphin, endomorphine-1, endomorphine-2, dynorphin, met-enkephalin, leu-enkephalin, galanin, and PAR-2 peptide.

21. Use according to any preceding claim, wherein the fusion protein comprises a purification tag.

22. Use according to Claim 21, wherein the fusion protein comprises a purification tag, which is present at the N-terminal and/ or C-terminal end of the fusion protein.

23. Use according to Claim 21 or 22, wherein the purification tag is joined to the fusion protein by a peptide spacer molecule.

24. Use according to any preceding claim, wherein the translocation domain is separated from the Targeting Moiety by a peptide spacer molecule.

25. Use according to any preceding claim, wherein the polypeptide fusion protein comprising any one of SEQ ID NOs: 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 52, 59, 61, 64, 67, 69, 71, 73, 76, 79, 82, 85, or 88.

26. A method of preparing a non-cytotoxic fusion protein, comprising:

a. contacting a single-chain polypeptide fusion protein as defined in any of Claims 1-25 with a protease capable of cleaving the protease cleavage site;

b. cleaving the protease cleavage site; and thereby forming a di-chain fusion protein.

27. A non-cytotoxic fusion protein, obtainable by the method of Claim 26, wherein the protein is a di-chain polypeptide, and wherein:

a. the first chain comprises the non-cytotoxic protease, or a fragment thereof, which protease or protease fragment is capable of cleaving a protein of the exocytic fusion apparatus of a nociceptive sensory afferent;

b. the second chain comprises the TM and the translocation domain that is capable of translocating the protease or protease fragment from within an endosome, across the endosomal membrane and into the cytosol of the nociceptive sensory afferent; and

the first and second chains are disulphide linked together.

28. Use of a fusion protein according to Claim 27, for the manufacture of a medicament for treating, preventing or ameliorating particular types of pain.

29. A method of treating, preventing or ameliorating particular types of pain in a subject, comprising administering to said patient a therapeutically effective amount of a fusion protein as defined in any of Claims 1-27.

Figure 1

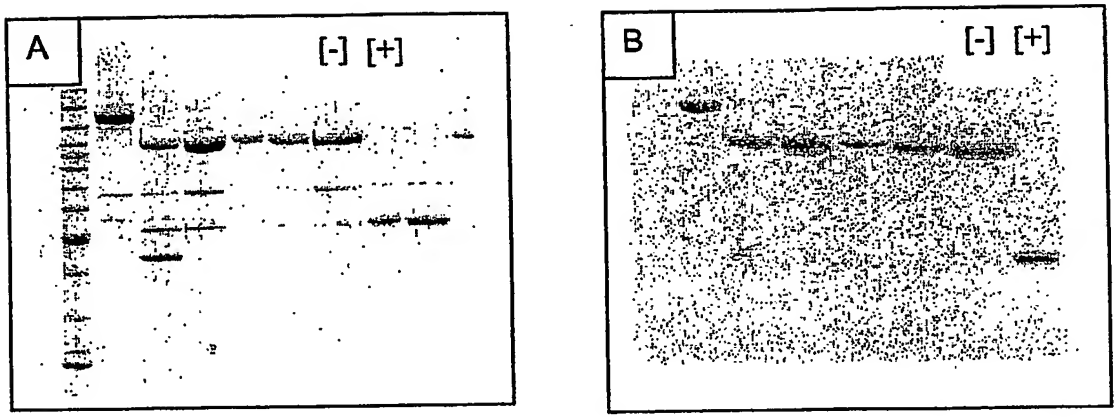


Figure 2

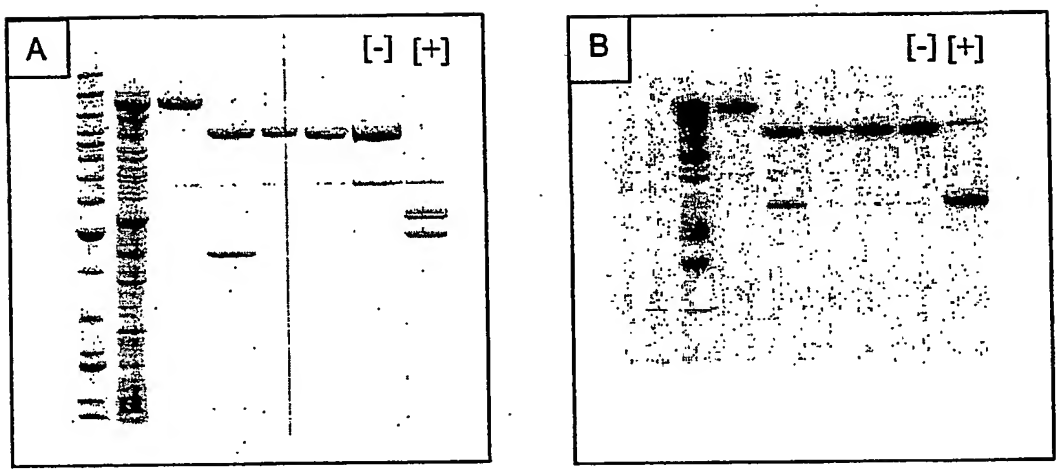


Figure 3

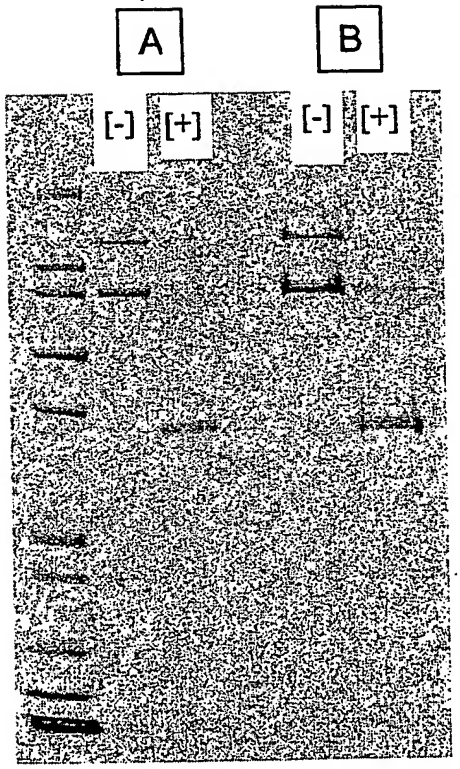


Figure 4

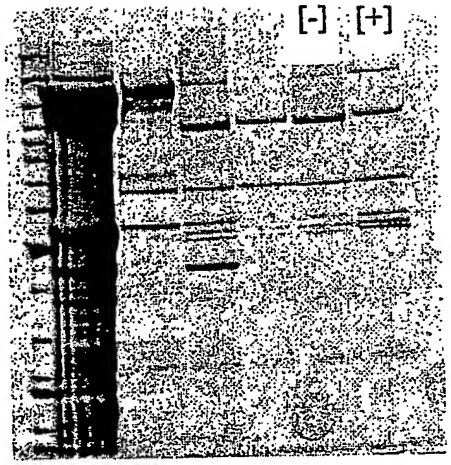


Figure 5

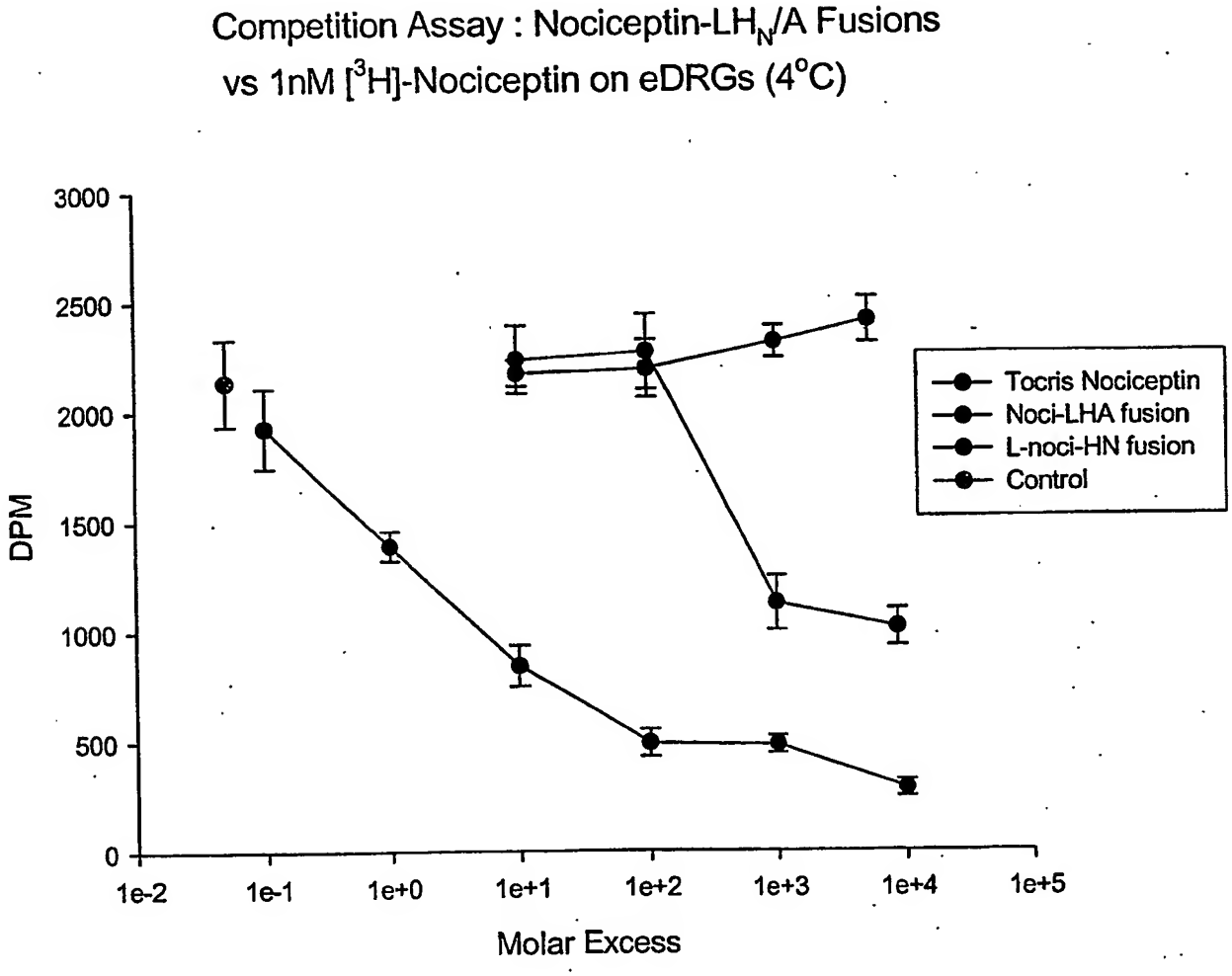

Competition Assay : Nociceptin-LH$_N$/A Fusions
vs 1nM [^{3}H]-Nociceptin on eDRGs (4°C)
DPM
3000
2500
2000
1500
1000
500
0
1e-2
1e-1
1e+0
1e+1
1e+2
1e+3
1e+4
1e+5
Molar Excess
Tocris Nociceptin
Noci-LHA fusion
L-noci-HN fusion
Control

Figure 6

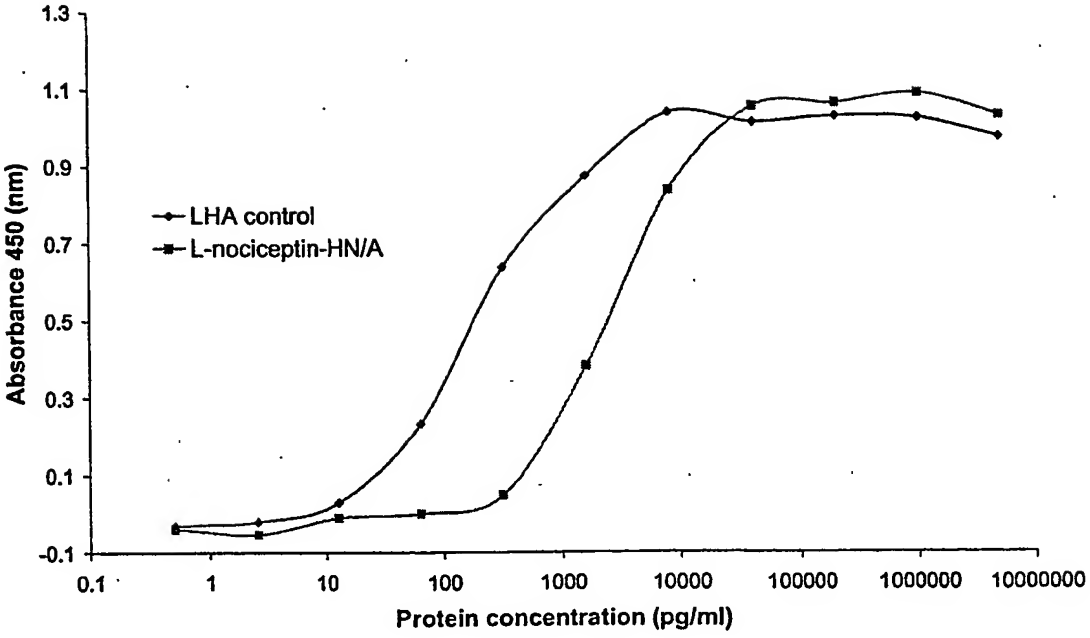

1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
Absorbance 450 (nm)
LHA control
L-nociceptin-HN/A
0.1
1
10
100
1000
10000
100000
1000000
10000000
Protein concentration (pg/ml)

Figure 7

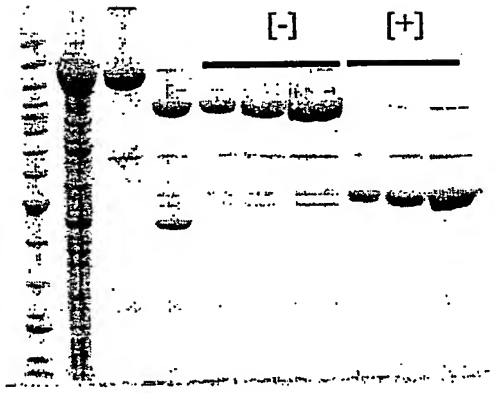


Figure 8

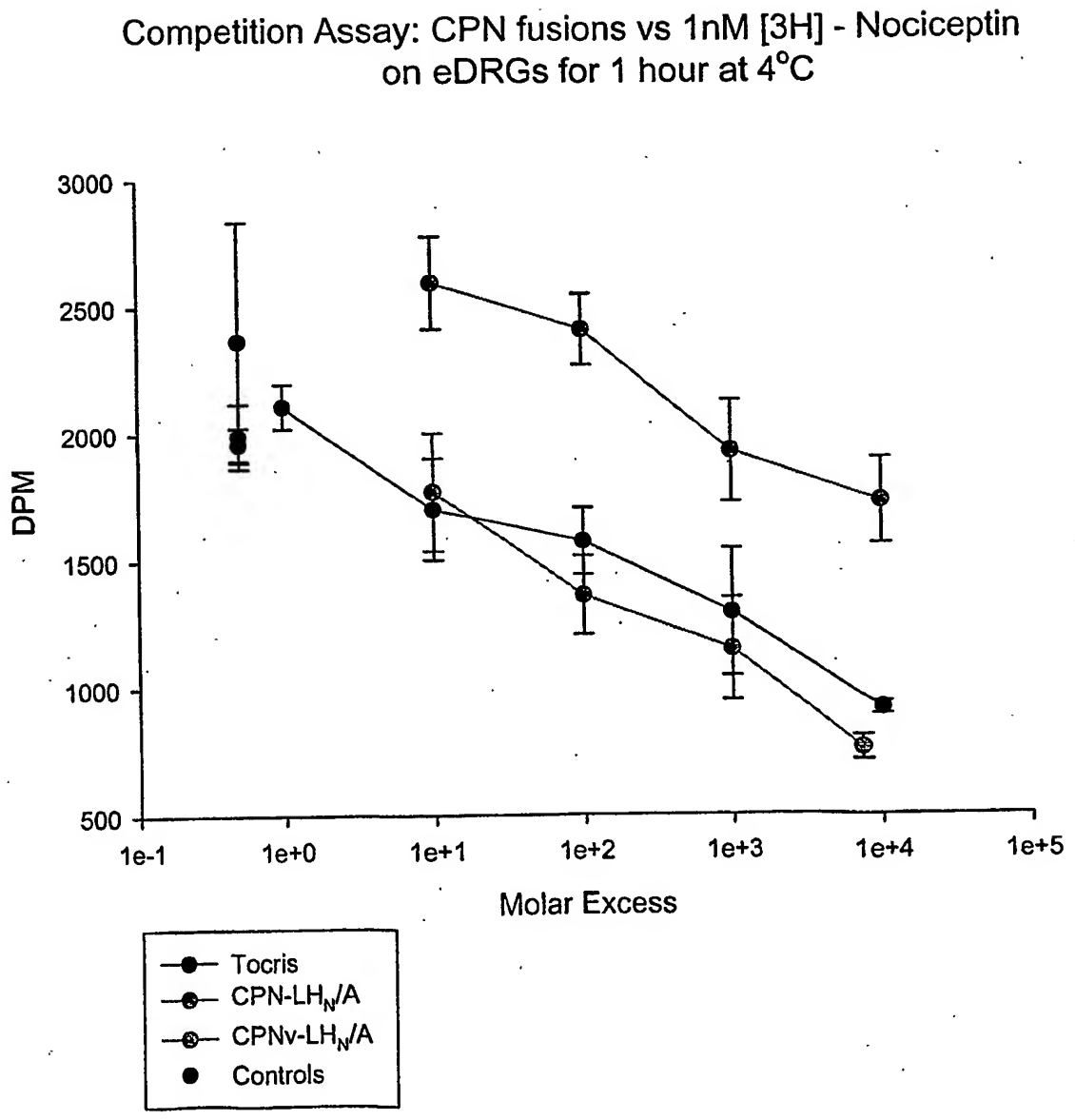

Competition Assay: CPN fusions vs 1nM [3H] - Nociceptin on eDRGs for 1 hour at 4°C
DPM
3000
2500
2000
1500
1000
500
1e-1
1e+0
1e+1
1e+2
1e+3
1e+4
1e+5
Molar Excess
Tocris
CPN-LHN/A
CPNv-LHN/A
Controls

Figure 9

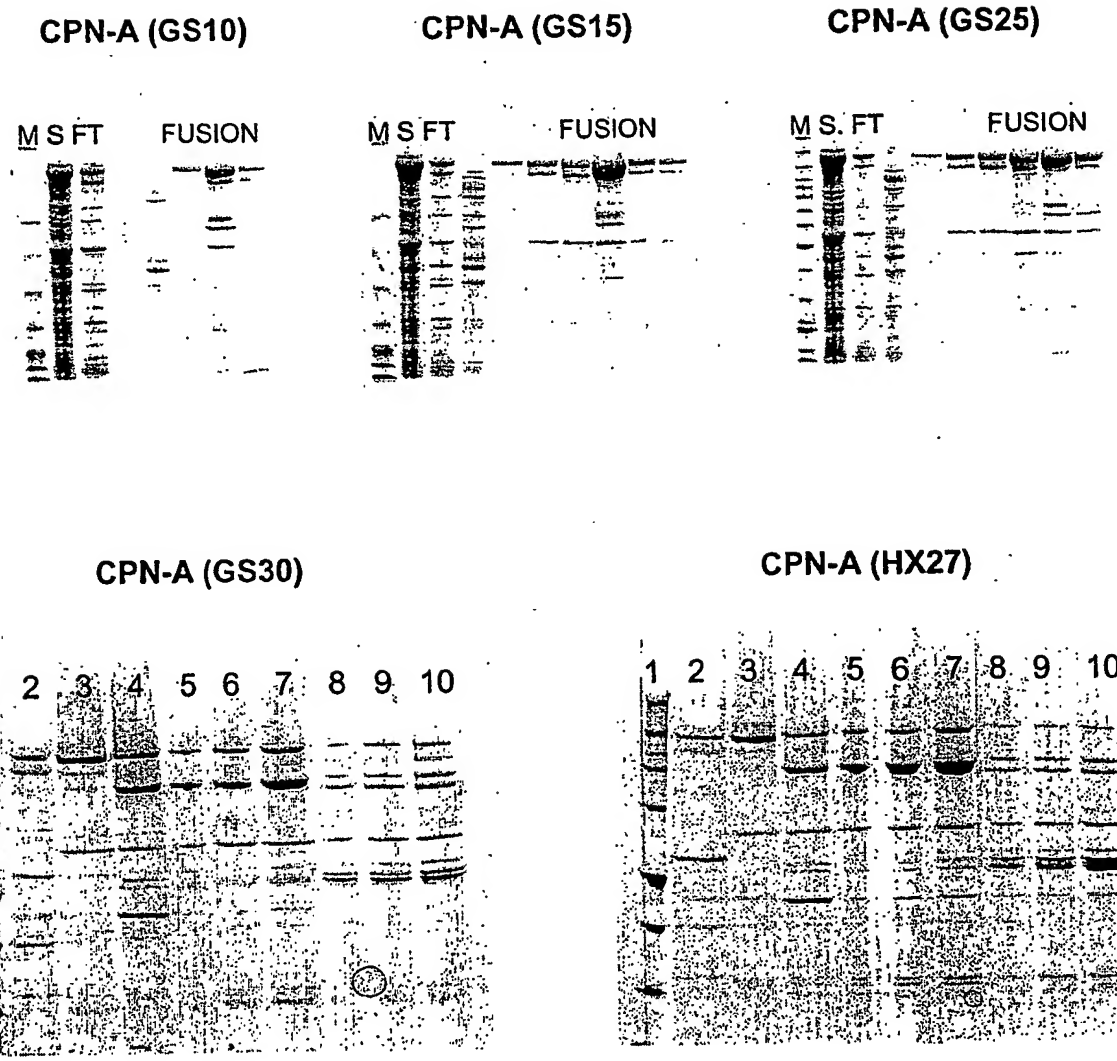

CPN-A (GS10)
M S FT
FUSION
CPN-A (GS15)
M S FT
FUSION
CPN-A (GS25)
M S. FT
FUSION
CPN-A (GS30)
1 2 3 4 5 6 7 8 9 10
CPN-A (HX27)
1 2 3 4 5 6 7 8 9 10

Figure 10

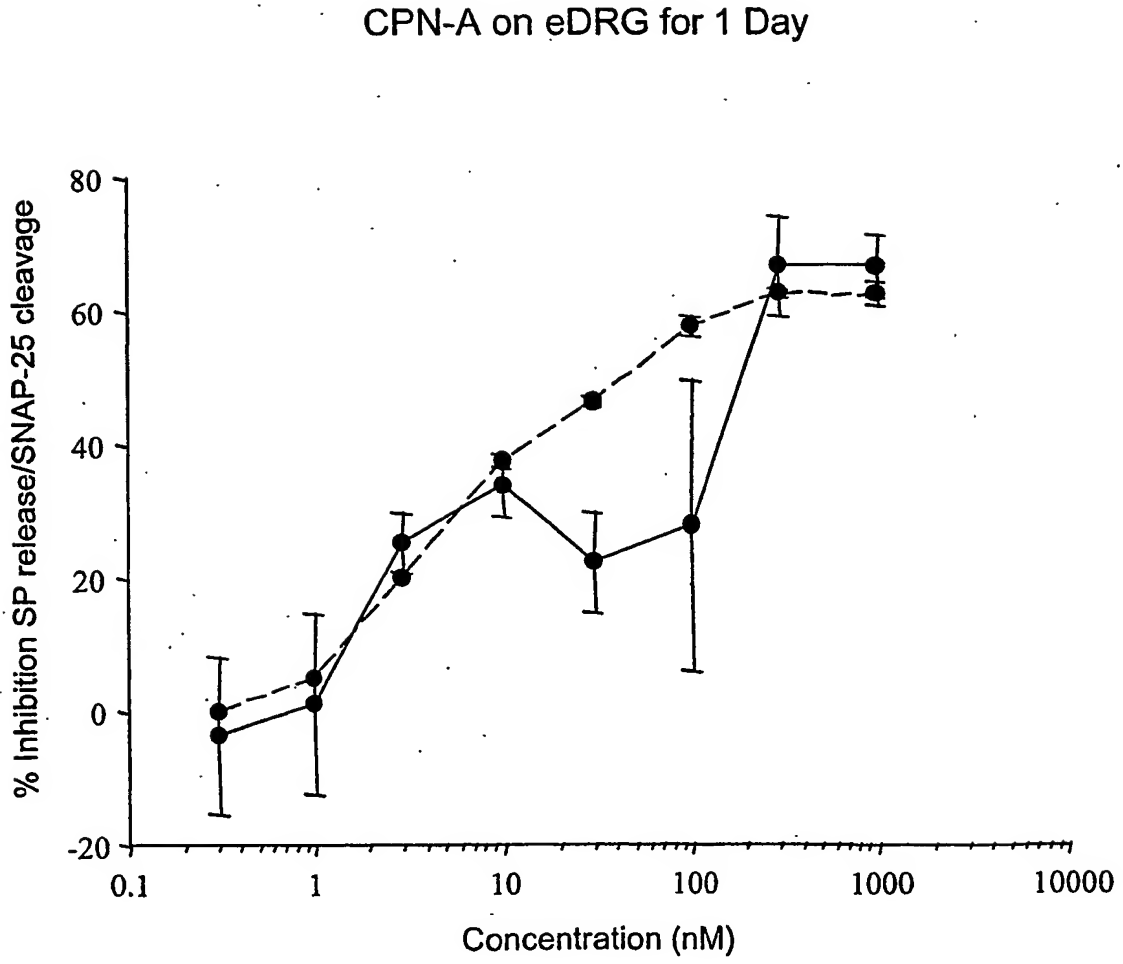

CPN-A on eDRG for 1 Day
% Inhibition SP release/SNAP-25 cleavage
80
60
40
20
0
-20
0.1
1
10
100
1000
10000
Concentration (nM)

Figure 11

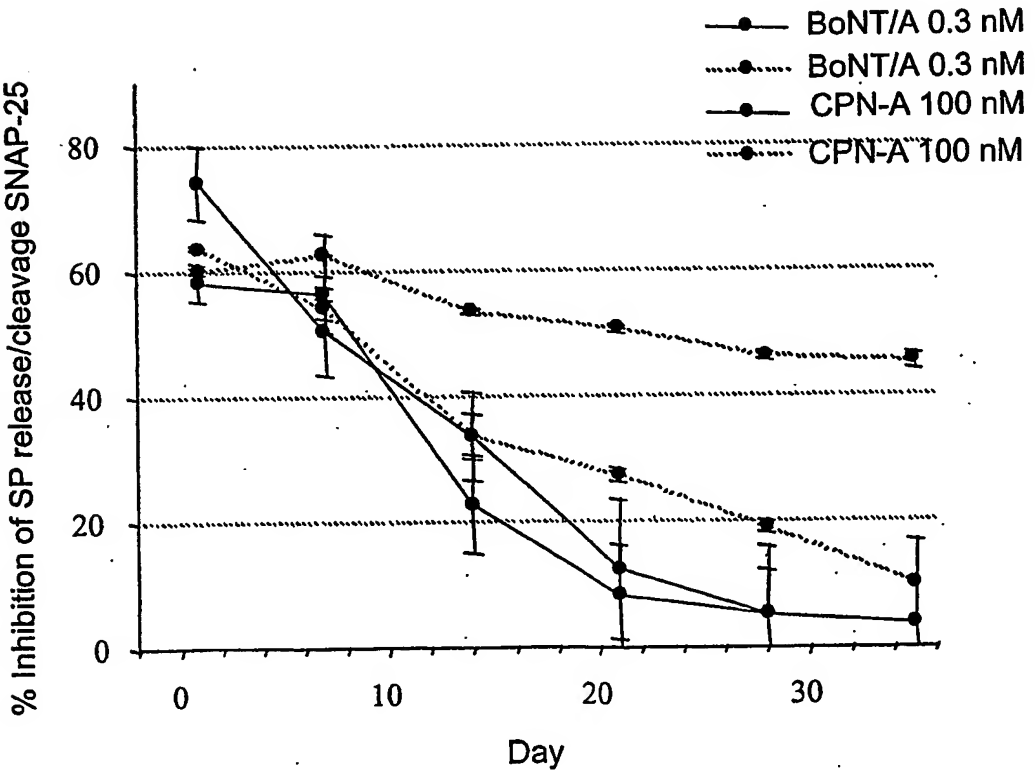

Duration of action following eDRG exposure for 1 Day
BoNT/A 0.3 nM
BoNT/A 0.3 nM
CPN-A 100 nM
CPN-A 100 nM
% Inhibition of SP release/cleavage SNAP-25
80
60
40
20
0
0
10
20
30
Day

Figure 12

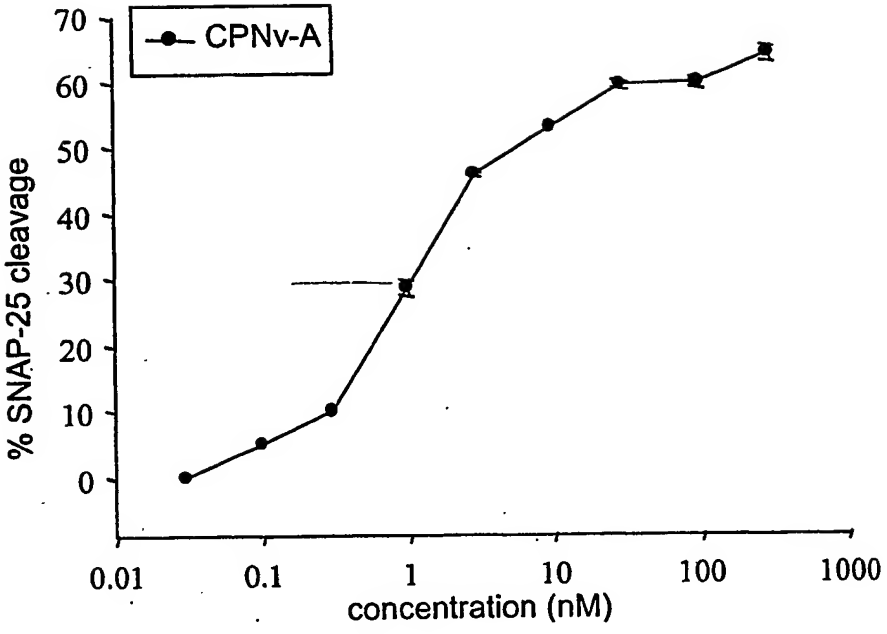

CPNv-A
% SNAP-25 cleavage
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
1000
concentration (nM)

Figure 13

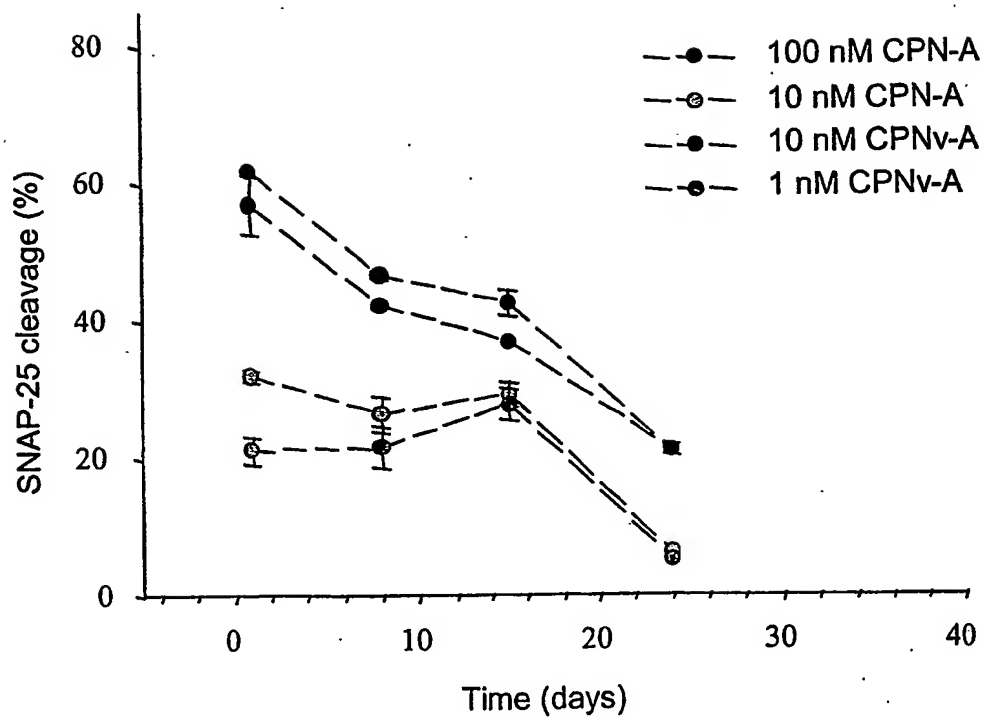

100 nM CPN-A
10 nM CPN-A
10 nM CPNv-A
1 nM CPNv-A
SNAP-25 cleavage (%)
0
20
40
60
80
0
10
20
30
40
Time (days)

Figure 14

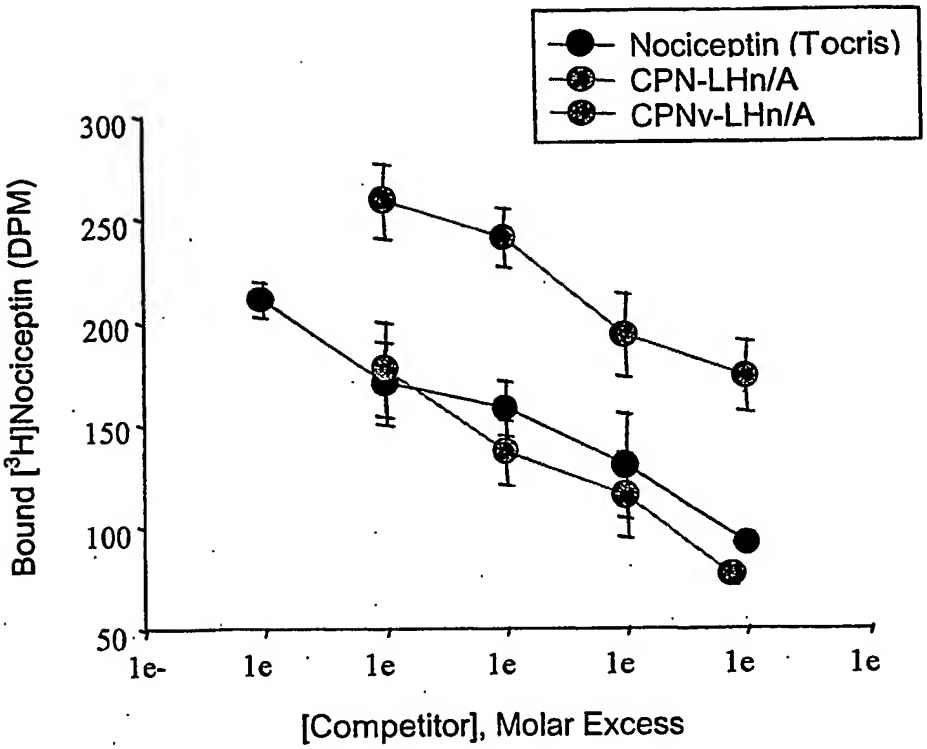

Nociceptin (Tocris)
CPN-LHn/A
CPNv-LHn/A
Bound [3H]Nociceptin (DPM)
300
250
200
150
100
50
1e-
1e
1e
1e
1e
1e
1e
[Competitor], Molar Excess

Figure 15

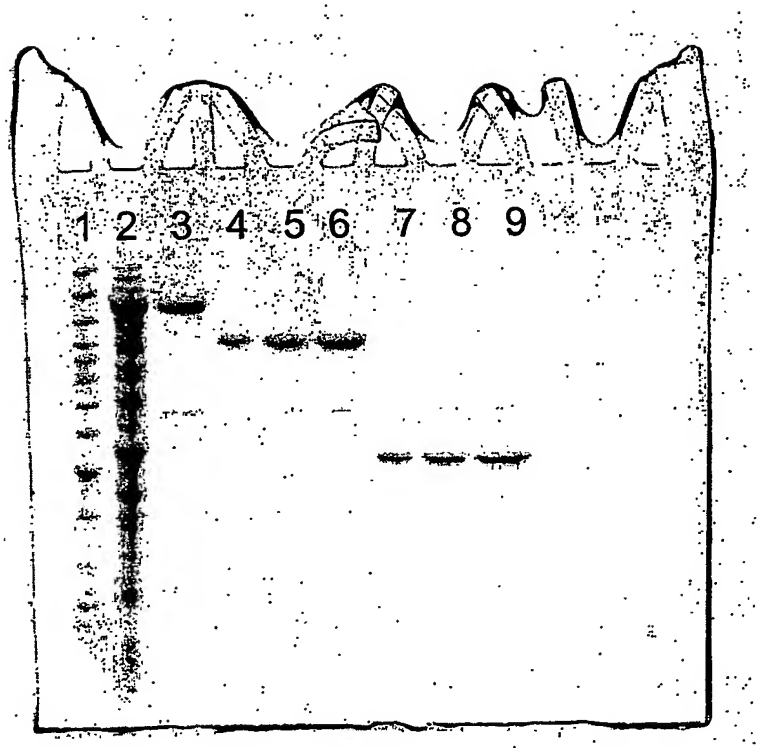


Figure 16

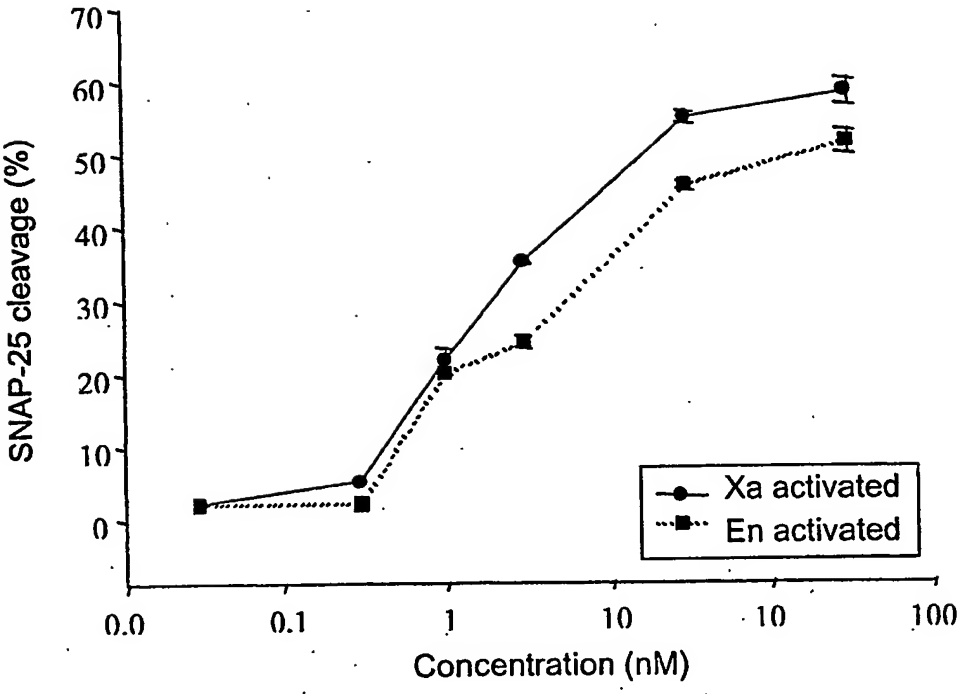

70
60
50
40
30
20
10
0
SNAP-25 cleavage (%)
0.0
0.1
1
10
10
100
Concentration (nM)
Xa activated
En activated

Figure 17

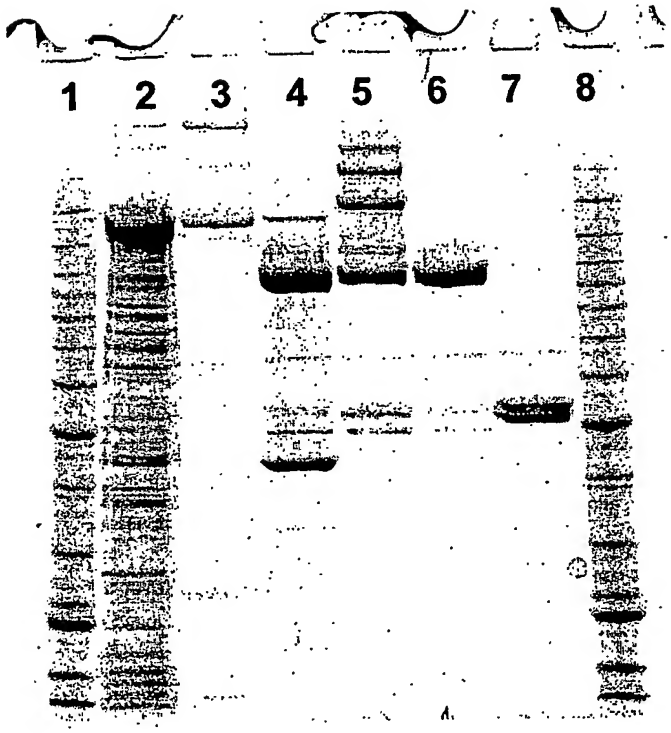


Figure 18

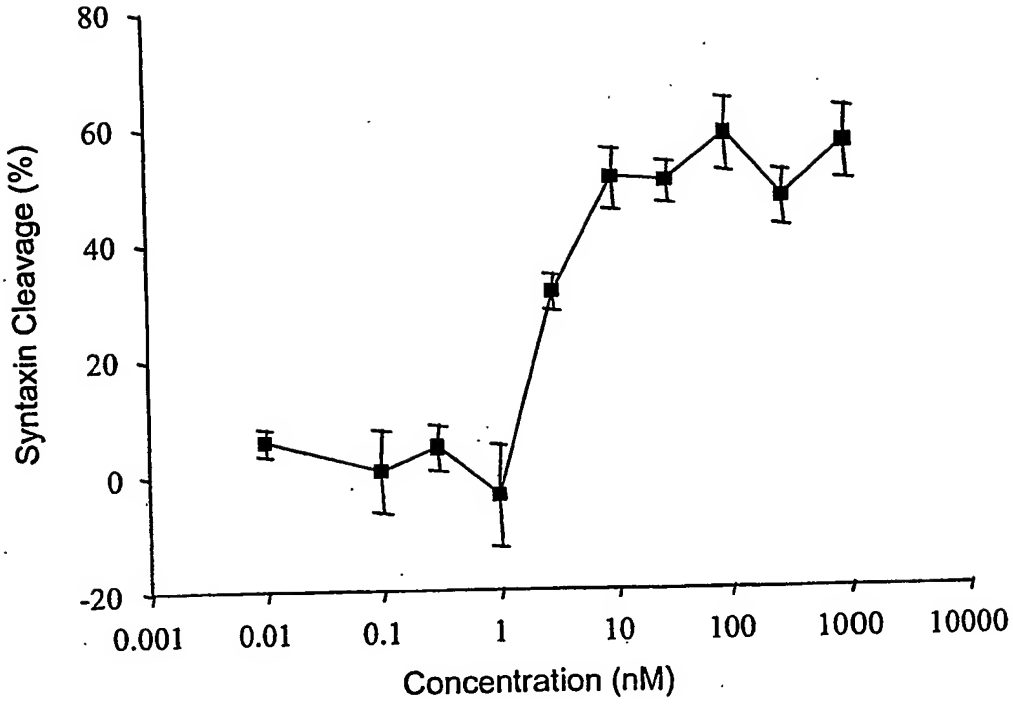

80
60
40
20
0
-20
Syntaxin Cleavage (%)
0.001
0.01
0.1
1
10
100
1000
10000
Concentration (nM)

Figure 19

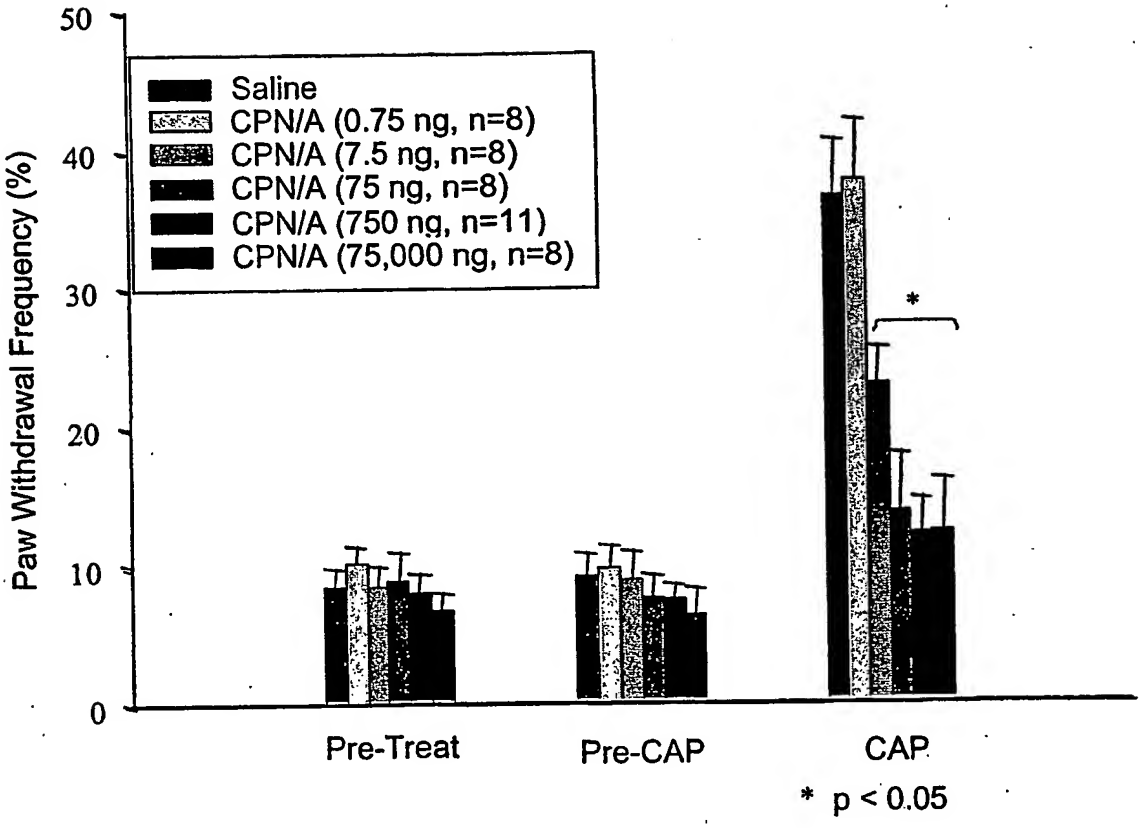

Paw Withdrawal Frequency (%)
50
40
30
20
10
0
Saline
CPN/A (0.75 ng, n=8)
CPN/A (7.5 ng, n=8)
CPN/A (75 ng, n=8)
CPN/A (750 ng, n=11)
CPN/A (75,000 ng, n=8)
*
Pre-Treat
Pre-CAP
CAP
* p < 0.05

Figure 20

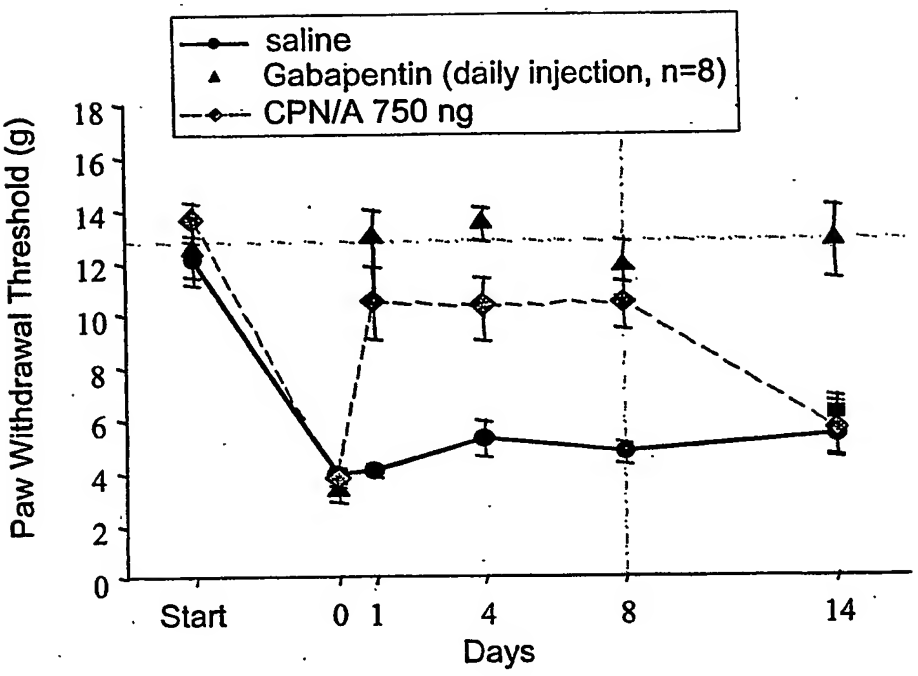

saline
Gabapentin (daily injection, n=8)
CPN/A 750 ng
Paw Withdrawal Threshold (g)
18
16
14
12
10
8
6
4
2
0
Start
0
1
4
8
14
Days

Figure 21

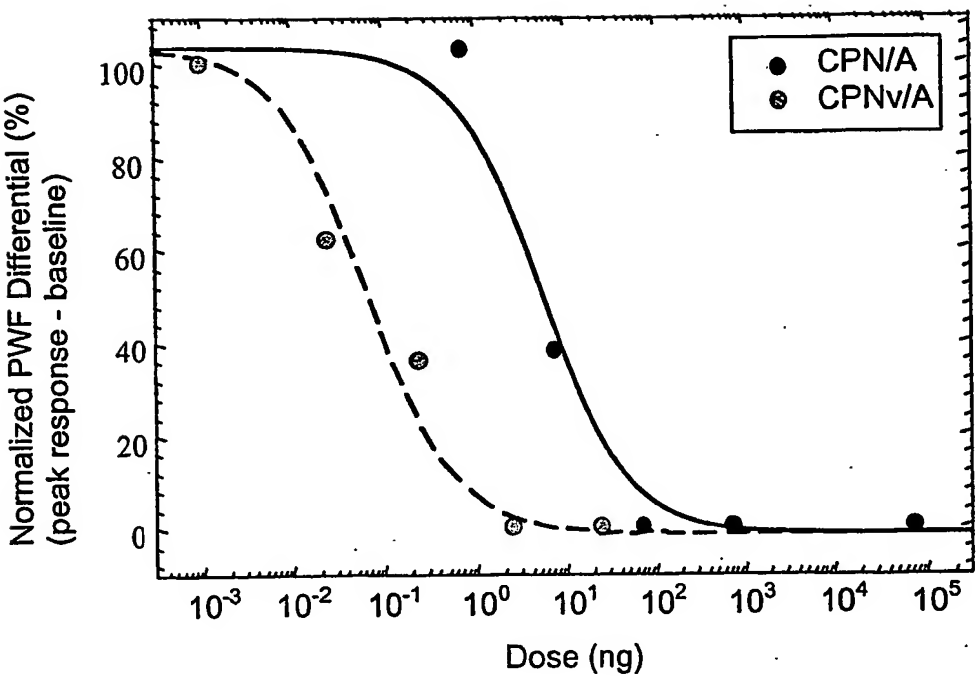

CPN/A
CPNv/A
Normalized PWF Differential (%)
(peak response - baseline)
100
80
60
40
20
0
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5}
Dose (ng)

Figure 22

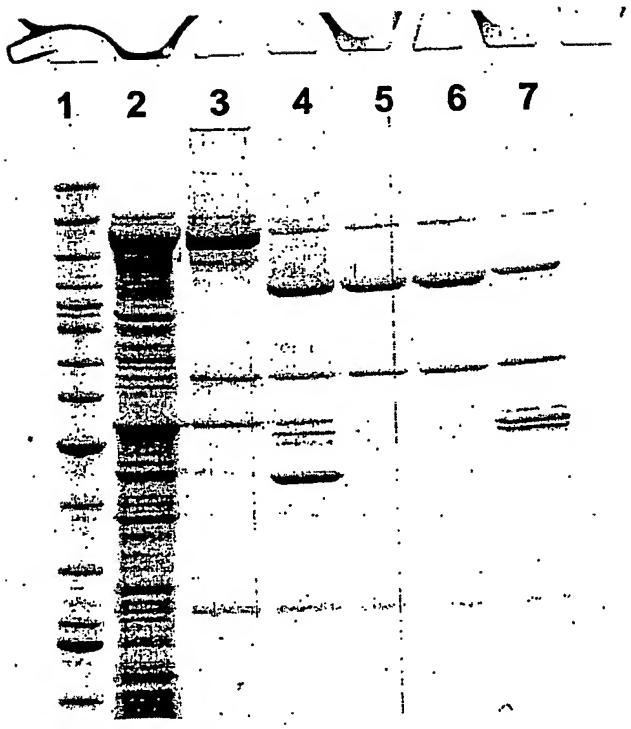


Figure 23

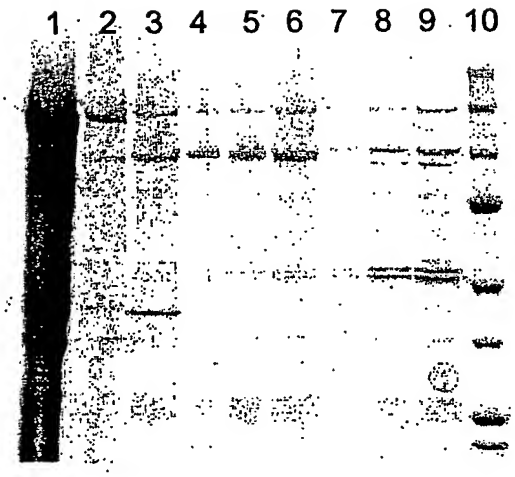


Figure 24

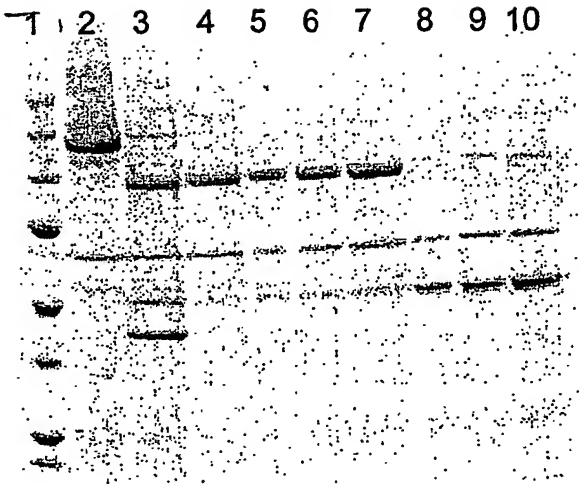


Figure 25

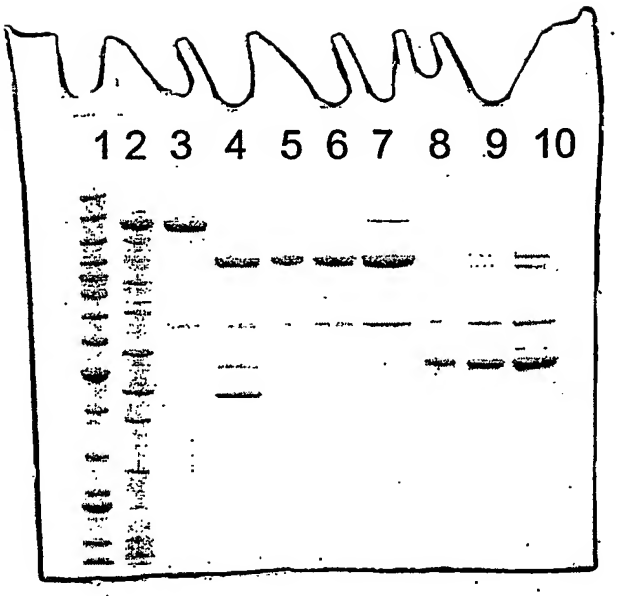


Figure 26

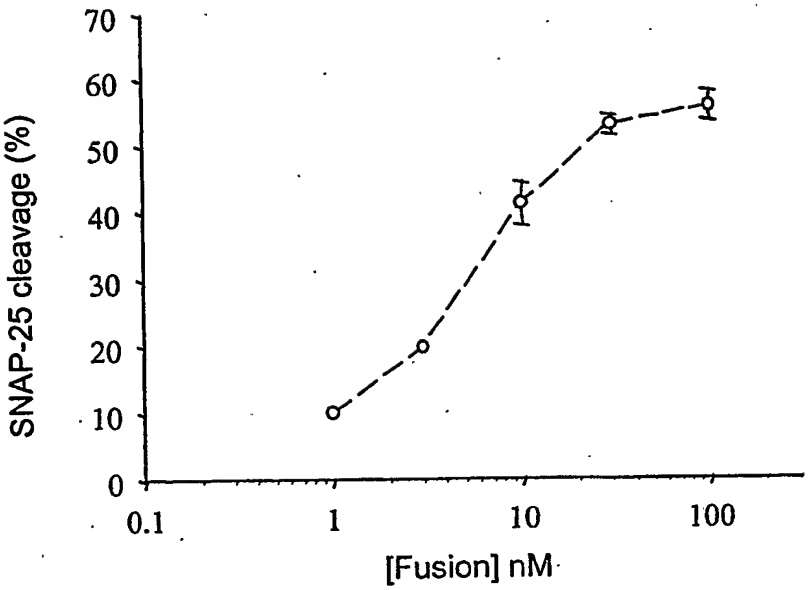
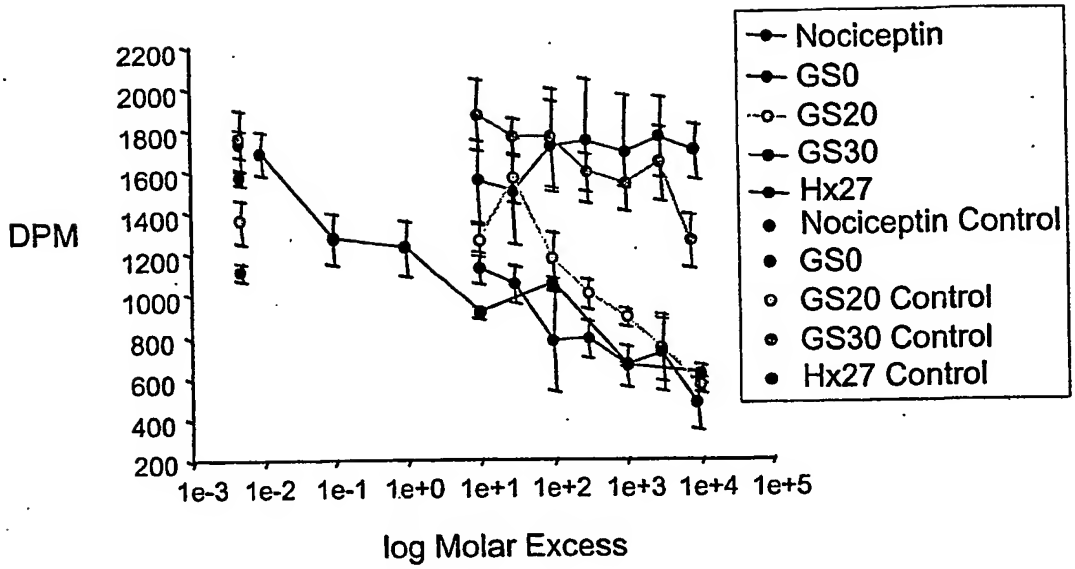


Figure 27

Figure 28


DPM
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
1e-3 1e-2 1e-1 1e+0 1e+1 1e+2 1e+3 1e+4 1e+5
log Molar Excess
Nociceptin
GS0
GS20
GS30
Hx27
Nociceptin Control
GS0
GS20 Control
GS30 Control
Hx27 Control

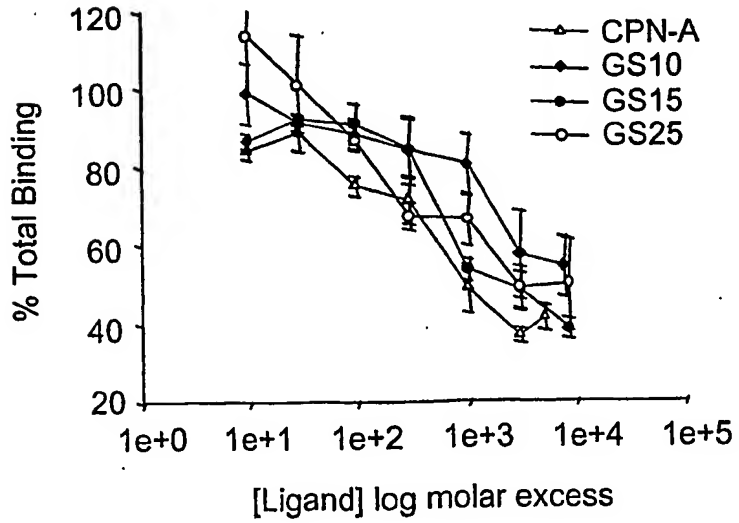
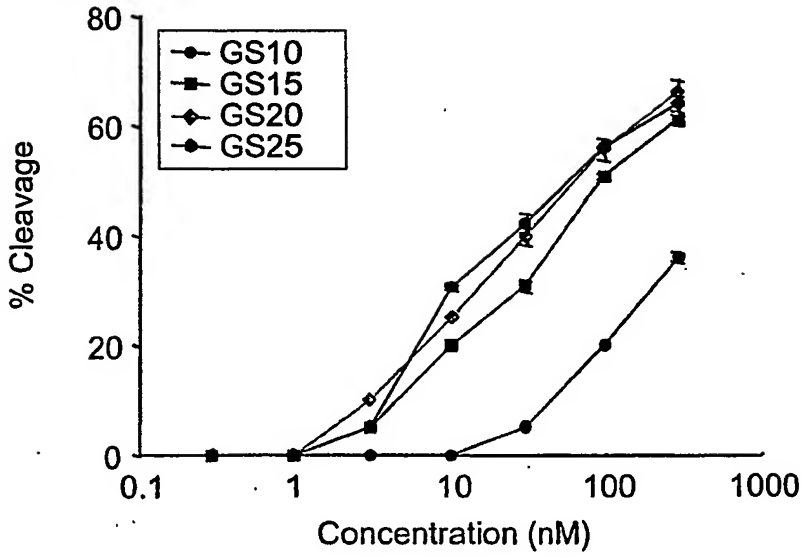

% Total Binding
120
100
80
60
40
20
1e+0 1e+1 1e+2 1e+3 1e+4 1e+5
[Ligand] log molar excess
CPN-A
GS10
GS15
GS25

Figure 29


80
60
40
20
0
% Cleavage
GS10
GS15
GS20
GS25
0.1
1
10
100
1000
Concentration (nM)